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**A BEHAVIOURAL INVESTIGATION OF THE FUNCTION OF
GLYCOPROTEIN FUCOSYLATION IN LEARNING AND
MEMORY IN THE DAY-OLD DOMESTIC CHICKEN.**

By

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A thesis submitted in partial satisfaction of the degree of Doctor of Philosophy.

The Brain and Behaviour Research Group

The Open University, Milton Keynes.

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LIST OF ABBREVIATIONS.

CS	- Conditioned stimulus
US	- Unconditioned stimulus
IMHV	- Intermediate medial hyperstriatum ventrale
LPO	- Lobus parolfactorius
PA	- Paleostriatum augmentatum
NMDA	- N-methyl-D-aspartate
HVc	- Hyperstriatum ventrale caudal (or Higher vocal center)
RA	- Round nucleus of the archistriatum
MeA	- Methylantranilate
M	- Methylantranilate trained chicks
W	- Water trained chicks
HPLC	- High performance liquid chromatography
2-Dgal	- 2-Deoxy-D-galactose
LED	- Light emitting diode
i.c.	- Intracranial injection
i.p.	- Intraperitoneal injection
LiCl	- Lithium chloride
Sal	- 0.9% saline
mol	- moles
sec	- seconds
min	- minutes
hr	- hours

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ABSTRACT

Two new paradigms of learning in the day-old domestic chick were developed and tested. The first was a serial colour-discrimination passive avoidance task, similar to that used by Gibbs and Ng (1977), in which chicks were trained to discriminate between red and green stimuli by pairing one with the bitter taste of methylantranilate. The second was a sickness-conditioned aversion task, similar to that developed by Garcia and Ervin (1968), in which chicks were trained to associate pecking at a stimulus with subsequent sickness, induced by lithium chloride injection.

2-Deoxy galactose (2-Dgal), a fucose analogue that inhibits the completion of brain fucosylglycoproteins, induced amnesia for both tasks when injected into both hemispheres of the forebrain before training. Amnesia was similarly induced for the passive avoidance task when 2-Dgal was injected into the right forebrain alone, but not the left forebrain. This indicates that fucosylglycoprotein incorporation has a more important function in the right forebrain after passive avoidance training.

No amnesia for the sickness-conditioned aversion task was detected after injection of 2-Dgal into either the left or the right forebrain alone. Thus either hemisphere was capable of learning this task but only one hemisphere was required.

2-Dgal was tested for a state-dependent action in both learning paradigms, by readministration of the drug before the retention test, in a 2×2 experimental design. The amnesia was not state-dependent, since readministration of 2-Dgal did not lead to recall of the learned response in either task. Thus the amnesia was not due to learned information becoming cue-dependent. Instead, amnesia was caused by the direct interruption of

molecular processes necessary for memory formation.

The results are discussed in the context of previous biochemical and morphological findings concerning fucosylglycoprotein incorporation and synaptic remodeling.

CHAPTER 1. GENERAL INTRODUCTION

Part 1. The significance of behavioural psychology to the study of memory.

For more than 20 years the study of the cellular mechanisms of memory and learning has been pursued by a multitude of workers, generating an enormous amount of literature on the subject (such as: Agranoff, et al 1976; Thompson, Berger and Madden IV, 1983; Thompson, 1986; Rose, 1985; Rose 1989). Perhaps the reasons for studying the mechanism of memory are obvious, however a reiteration of those reasons here would not be redundant.

Why is the study of memory so important? Memory is the property of the nervous system that sets it apart from any other mass of living tissue, since no other tissue can retain and process complex information. Incoming information is acquired, remembered and recalled at the appropriate time. It is of obvious importance from a clinical point of view to study the processes of acquisition and retrieval in order to bring aid to people suffering from learning deficits such as amnesia, dyslexia or Alzheimers disease. However, acquisition and retrieval of information, separated by long periods of storage, are in effect, the processes that we find convenient to study experimentally. Even more important than these processes is the retention of short term memories, within the conscious arena of awareness. Without such memory there would be no possibility of reference to previous events (no matter how recent those events were). Thus there would be no way to interpret new information, since interpretation is only possible in the context of preceding events. The reader can only make sense of the present sentence when it is read in the context of the previous one. Without the memory of the last sentence, reading

(and understanding) would be impossible. Without memory there would be no way of making sense of what we perceive from one minute to the next. So, it seems, memory is the fundamental mechanism by which the nervous system operates. In studying this mechanism we are in fact, discovering the mechanism of the most fundamental attribute of the nervous system.

Since this thesis takes a behavioural, and therefore a holistic approach to the problem of memory it is important at this stage to justify such an approach. The behavioural approach must attempt to conduct experiments to satisfy criterion 4 of those criteria necessary, sufficient and exclusive for identifying the biochemical correlates of memory formation stated by Rose, that is, "If the cellular/biochemical changes.... are inhibited during the period over which memory formation should occur, then memory formation should be inhibited, and vice versa." (Rose, 1981, p.814). Thus the chief role of the behavioural pharmacologist in the study of memory is to experimentally inhibit those biochemical correspondents and observe that inhibition does indeed cause amnesia.

The bi-product of a multidisciplinary approach to brain function is the analysis of the problem at many levels, from the molecular to the behavioural; of those levels of analysis, the least microscopic (and therefore the most holistic) is that of the behavioural approach. Translation of observations between one level and another sometimes seems more difficult to achieve than the actual research needed to make those observations. This is partly due to philosophical differences between the different disciplines, but is mostly due to "language" differences.

Because of the different technologies used to address the problem of memory, there have evolved different ways of defining the problem, and consequently it is necessary that

there are different languages needed. The electrophysiologist speaks of memory in terms of trains of coded action potentials, while the biochemist talks of memory in terms of chemical cascades giving rise to changes in the chemical nature of neurons (Rose, 1988). These idiosyncratic views of causality are necessary, if only because of the complexity of the technology used to measure the results of memory retention. What is needed is a coordinating discipline to act as interpreter. In a sense, this is a justifiable goal for the behavioural psychologist, who is (theoretically) in the ideal position to integrate the observations from all other disciplines. In which case, the role of behavioural psychology is that of integrating, interpreting and testing the observations of other disciplines.

Having assumed this rather weighty responsibility, however, the psychologist is left to study the behavioural expression of the synthesis of all memory related phenomena. An animal expresses a behavioural response that will indicate recall (or partial recall) or amnesia; the chick either pecks at a bead or avoids it. The end result of integrating the many complex mechanisms of the brain is a rather easily described behavioural response. It is all too easy for the experimental psychologist to ignore the neural mechanisms that are operating to produce the behaviour. The "black box" approach is assumed and the behavioural approach becomes purely descriptive, explaining the behaviour in terms of external factors only.

In performing experiments on the behaviour of animals, I wish to use those findings to move towards a synthesis of all other findings concerning the neurobiological mechanism of learning. Although this goal probably remains well out of reach of human endeavor, the possibility of some reasonable approximation remains.

Of course, to address such complex questions we must study equivalent mechanisms in systems more simple than the human. There follows a short review of some of the

"model" systems that are currently being used to study memory. Following this, part 3 of the introduction deals more specifically with learning and memory in birds, a field of neuroscience that has emerged over the last 30 years and is still developing. Part 4 considers the behavioural aspects of the young chick as an experimental animal in the study of memory. The ethology of the chick makes it ideal, in some ways, for studying memory and learning, but some aspects of its behaviour tend not to be considered in the design of learning experiments. Finally, part 5 reviews the literature concerning the brain glycoproteins that have become of interest to memory and learning research .

Part 2. Models of Learning

i) Invertebrates

A number of invertebrate species have been used as models for studying the biochemical correlates of memory formation (for review see Nelson and Alkon, 1989). These approaches have tended to concentrate on biochemical events within either the pre- or post-synaptic bouton, concurrent with a reflexive, or at best a simple associative response to mechanical stimulation. Invertebrates have the advantage of being relatively simple organisms with a well mapped nervous system. The neural network responsible for the gill and siphon withdrawal reflex in the marine mollusc *Aplysia californica*, has been mapped (Kennedy and Davis, 1977) and it is now possible to extract part of this neural network and re-connect it in vitro, thus enabling highly controlled experiments on the cell biochemistry of the network.

Kandel hypothesised that the basis of short term habituation in aplysia was caused by the passage of calcium ions across the neuronal cell membrane (Bailey and Kandel, 1985). In

his model, an action potential stimulates the opening of calcium channels, allowing calcium to enter the cell and causing synaptic vesicles to bind to presynaptic zones, releasing transmitter substances. Repeated stimulation during training causes a reduction in the number of open calcium channels. Long-term habituation is accomplished by structural changes such as a decrease in the number of active varicosities in nerve cells, along with an increased strengthening of other connections.

By using *Aplysia* as a model of memory, Kandel and others have greatly advanced our understanding of the biochemistry of the neuron. Certainly, much is now known about the possible ways in which transmission between synapses may be facilitated and a number of biochemical schema have been proposed. This approach however, has the disadvantage of being a very simple model of learning and may not reflect the true nature of learning in more complex organisms, in spite of claims that memory can be encoded in single neurons (Gingrich and Byrne, 1987). Alkon (1983) made the point that behaviour is more likely to be determined by the way in which the nervous system as a whole is organised, rather than the properties of individual cells within that nervous system.

There is also an associative paradigm for the *Aplysia* in which a strong shock to the tail (US), producing a very large gill withdrawal response, is paired with a weak mechanical stimulation of the siphon (CS), that normally produces a mild response. When the CS and US have been paired within 30 secs of each other for 20 to 30 trials, a presentation of the CS alone produces a powerful gill and syphon withdrawal (Lukowiak and Sahley, 1981; Carew, Hawkins and Kandel, 1983).

Another invertebrate model used to study simple learning is the marine mollusc, *Hermisenda crasicornis*. In normal conditions the animal will display phototactic

movement, approaching a light source, however after exposure to the light (CS) is paired with rapid rotation (US), a stimulus that normally initiates foot contraction, the animal eventually exhibits foot contraction in response to light stimulation (Crow and Alkon, 1978). This is possibly a more realistic model of classical conditioning since the CS and US are of different sensory modalities, although the behaviour may have little biological significance.

In some ways the models of learning described above are ideals, since the behaviour being studied is very simple and it is much easier to relate the changes found in the simple nervous systems of these animals to the behaviour that has been observed. However, there is a danger in stressing too heavily the findings of this type of work, since the properties of the nervous system of *Aplysia* are very far removed from those of more advanced organisms. Beyond indicating how individual neurones behave in a simple learning network, the study of marine molluscs will reveal little of the way complex behaviour emerges from the brains of higher organisms.

In the search for compromise between studying brains comparable to the human and neuroanatomical simplicity, some workers have turned to insects for their experimental subjects. Because of their simple nervous systems the insects are relatively easy to study, however, they possess a central ganglia representing a simple brain. An associative learning paradigm has been developed with the fruit fly, *Drosophila melanogaster*. The normal (or wild-type) *Drosophila* readily learns to associate shock with a distinctive odour in a multi-trial avoidance task. The task usually takes the form of a discrimination between two distinctive odours, one associated with shock and the other with reward. Good retention of discrimination learning has been observed for 5 hr and more (Quinn et al, 1979).

Genetic mutations of *Drosophila*, incapable of learning the odour discrimination task, have been studied by Dudai (Dudai et al, 1976). Four different mutations, each incapable of performing the task, have been isolated and called *Dunce*, *Turnip*, *Cabbage* and *Rutabaga*. There is also a mutant called *Amnesiac*, that can learn normally but shows impaired retention 1 hr after training. Analyses of the different mutants have established well defined metabolic defects. *Dunce*, for instance, has abnormally high levels of cAMP (Byers et al, 1981), while *Rutabaga* has abnormally low cAMP levels (Livingstone and Temple, 1983).

As with the marine mollusc paradigms, the various genetic defects of *Drosophila* give some idea of the biochemistry of the insect nervous system and the findings may relate to neural mechanisms that are similar for all species. However, little can be learned about the organisational properties of more advanced brains by studying such small brains.

A second insect that has been studied in relation to learning is the honey bee, *Apis mellifera capensis*. The bee is held in a small yoke and its proboscis is stimulated in a paradigm similar to that of the *Aplysia* model. The CS of odour or colour is used with a US of sucrose dripped onto the proboscis. The bee can learn a discrimination between two odours such as carnation and orange with great accuracy. Some pharmacological studies have been carried out and a certain degree of functional localisation has been realised within the bee brain (Menzel and Michelson, 1986; Brandes, Frisch and Menzel, 1988).

Although much has been achieved using this simple learning paradigm, the findings have yet to be confirmed in more complex examples of bee learning. The model shows great promise for future development. Honey bees are highly efficient foragers capable not

only of remembering the source of new food but also of communicating this information to other members in the hive (Bitterman et al, 1983; Menzel, 1985). It should soon be possible to develop a more biologically appropriate model of learning using the honey bee. Although we should not belittle the what has recently been achieved by studying the bee brain, a great deal more could be achieved by studying the bee in a more elaborate and biologically appropriate paradigm.

ii) Fish

The goldfish (*Carassius auratus*) has been used for many years by Shashoua to investigate neurochemical changes in the fish brain associated with learning. The task involves attaching a small float to the ventral thoracic area of the fish, which has the effect of disrupting swimming coordination. The goldfish learns to compensate for the action of the float within 5 hr (Shashoua and Moore, 1978) and this behaviour has been demonstrated to be accompanied by increased secretion of acidic glycoproteins called ependymins, into the cytoplasm and cerebrospinal fluid of the fish central nervous system (Shashoua, 1976). How this paradigm relates to learning in a more natural setting has never been clear to me. It is unlikely that a goldfish would normally need to adapt its swimming behaviour to compensate for such a large change in its orientation. The behavioural task probably involves a large amount of stress and an increase in motor activity on the part of the goldfish. It is possible the reported increase in ependymin secretion is due to other aspects of the task apart from learning. Indeed, it is not clear what in fact the goldfish is learning in this task. Higher cognitive processes may not be involved at all since the change in behaviour is reminiscent of the acquisition of a new motor skill.

More recently, similar results have been obtained using a classical conditioning paradigm

(Shashoua and Hesse, 1988), in which the fish is trained to associate a light (CS) with the onset of electrical shock (US). Replication of the earlier results using this paradigm suggests that endymnin secretion is in fact a brain correspondent of learning, rather than the effect of other aspects of the task.

Goldfish endymnins have also been implicated in a shock-avoidance paradigm (Piront and Schmidt, 1988). Fish were trained to cross a barrier in order to avoid electric shocks applied shortly after the onset of a warning light. Injection of anti-endymnin antisera from 0.5 to 24 hr after training caused amnesia for the task, indicating that the anti-sera interfered with the formation of long-term memory and further implicating endymnins in goldfish memory.

iii) Mammals.

Small mammals, such as rats and mice, tend to be favoured in the neurobiological approach to learning, since experimental intervention is relatively easy and ethical. Small mammals are also available in large quantities so their use makes replication of experiments possible. Described below are a few examples of those vertebrates used in the neurobiological approach to the study of memory, along with description of the many different learning paradigms.

Rats particularly, have been highly favoured in the study of memory. In many cases active and passive avoidance paradigms are employed in training small mammals. These tasks usually involve escape from some type of punishment such as foot-shock, and are learned in a small number of trials, thus making it possible to kill the animal soon after learning and assay putative biological changes within the brain. Avoidance tasks have

been used as learning paradigms in the rat (Morgan and Routtenberg, 1979), the mouse (Izquierdo and Mc Gaugh, 1987). Alternatively a brightness discrimination task has been used, in which the subject must learn to enter a lighted compartment in order to avoid a punishment (Wetzel et al, 1980; Popov et al, 1983).

Numerous operant learning tasks have also been used with rodents, in which the animal must change some aspect of its surroundings in order to receive a reward. This type of task has been used extensively, not only with rats (Perin, 1943) but also with pigeons (Irwin, Barraco and Terrian, 1978; Brown and Jenkins, 1968; Macphail and Reilly, 1985), chicks (Mc Dougall, Zolman and Mattingley, 1987) and many other species including cats, dogs and primates (see Bitterman, 1975). The operant conditioning approach has, however, become unfashionable in neurobiology because of the many trials required for learning to occur, the lack of biological adaptiveness of most of the learned information and the lack of generality across species in these tasks (Seligman, 1970).

Other tasks that have been used to study the neurobiological correlates of behavioural changes may not be considered to be truly associated with learning. One such paradigm is the comparison of brains from rats raised in complex and impoverished environments. Rats raised in home cage environments containing numerous small toys of different colours had more synapses per neuron in the visual cortex, compared to those raised in normal, sparse conditions (Greenough, Hwang and Gorman, 1985). These changes were also accompanied by greater population excitatory post-synaptic potentials and granule cell population spikes, in rats raised in the complex environment (Green and Greenough, 1986). There is the question of whether results from this task are due to developmental differences between the two groups rather than true learning. However, the results raise

an important question of how experimental animals should be housed, since the normal laboratory conditions tend to be rather sparse and uniform. It could be that the laboratory animal is adversely effected by these conditions, in which case, behavioural experiments will tend to be carried out on laboratory animals with underdeveloped brains.

Another unusual learning paradigm is the sickness-conditioned aversion task, in which sickness is induced by injecting a poison, some time after the subject has consumed a novel coloured or flavoured food. This type of learning has been studied in rats and many birds (Wilcoxon, Dragoin and Kral, 1971). Sickness-conditioned aversion in chicks will be discussed in more detail in chapter 3.

The attraction of tasks such as sickness-conditioning, passive avoidance training and maze learning is that the tasks are highly representative of learning taking place in the wild situation. Learning takes place quickly and is adaptive, providing the animal with information necessary for survival. It is these attributes that have made such tasks so popular for the study of the neuronal correspondents of learning.

One particular ability of the rat, that of foraging for new food in a highly efficient manor, has been exploited in the design of the radial-arm maze (Olton, 1987). Rats are able to remember with great accuracy the types of food that are safe and also where those foods were last obtained. The radial-arm maze utilises these foraging abilities in its design. The rat has a choice of eight or more corridors radiating from a central compartment, each corridor containing a small food reward at its end. If the rat is placed in the center of the maze it will visit each arm in turn and recover the reward, visiting each arm only once. To navigate, it uses visual cues such as the walls and ceiling of the lab. Thus it remembers a cognitive map of the maze in relation to the rest of its surroundings and operates on this information each time it removes food from one of the arms, maximising the efficiency of

its foraging behaviour. There have been variations on this general paradigm, such as odour discrimination (Staubli, Baudry and Lynch, 1985). It is thought that the processes of spatial memory may be different to those of avoidance learning (Staubli, Baudry and Lynch, 1984). It has been demonstrated that the hippocampus is of fundamental importance in this type of task (Olton et al, 1986; O'Keefe and Nadel, 1978).

Work using the radial-arm maze lead Olton to postulate that two types of memory exist, operating independently of each other. The first, called working memory, encodes information concerning specific and temporal aspects of a situation and is dependent on the hippocampus. The second, called reference memory, is responsible for coding general information concerning rules and procedures specific to a task. This memory is independent of the hippocampus but may have a locus in the neocortex.

A similar spatial task, in which a rat is placed in a deep bath of cloudy water and must learn the position of a submerged platform, has been used by Morris et al (Morris et al 1986; Morris, Hagan and Nadel, 1987). Again, the rat has been shown to be capable of performing this task by using external cues (reference memory), or by its own body movements (working memory).

Many workers following the cognitive approach postulated similar memory models, in which two types of memory were utilised in parallel, yielding a number of separate theories and terminologies. However, Kesner suggested that it was possible to form one model by integrating the many theories (Kesner, 1986) in which the two types of memory were referred to as "data-based" and "expectancy-based" memory. The model encapsulated many of the other theoretical frameworks in which a dualistic terminology was used to describe two types of memory. Examples of such are: Olton's working and

reference memories; Tulving's episodic and semantic memories; Mishkin's recognition and habit; Squire and Cohen's declarative and procedural memories and O'Keefe and Nadel's taxon and locale system.

Similarities can also be found amongst clinical observations of the nature of memory, as far back as the previous century. William James (1890) distinguished between primary and secondary memory, these were further classified as short and long term memories, postulated by Broadbent (1958) after the observations of HM, a patient who had been given extensive hippocampal lesions to prevent epilepsy. HM could recall pre-operation events quite accurately but had great difficulty encoding new working memory. He was capable of learning new mechanical skills but could not report having learned those skills.

The idea of short and long term memory was generally demonstrated by Hebb, who asked college students to repeat strings of digits. He found that most students had difficulty in remembering a string of more than eight digits, unless that string was repeated several times, indicating that short term memory had a finite capacity and would lose information if that capacity was exceeded. However, once information in short term memory was repeated it became encoded in long term memory and would be stored for a much longer time.

Hebb developed a theory to explain memory in neurobiological terms (Hebb, 1949). He argued that cells must be connected in reverberating circuits in order for short-term memory to be stored as transient activity in the brain. Furthermore, Hebb postulated that permanent structural changes must take place within those circuits for the maintenance of long-term memory.

Hebb's ideas are still popular today and much of the neurobiological evidence reinforces

them (Kuroda, 1989). Invertebrate work has also led some to postulate single synapse models of learning (Gingrich and Byrne, 1987; Stent, 1973; Gluck, Parker and Reifsnider, 1988). These models emphasise that plastic changes in the synapse must be responsible for the storage of long term memory. Even this reductionist view point does not necessarily conflict with the Hebbian model, under the condition that changes at a single synapse are interpreted in the context of effecting a change in the whole cellular network, rather than being independently responsible for encoding a memory.

The cognitive approaches to learning in mammals have demonstrated that there is generality between animal models of learning and clinical observations, thus findings from animal models should ultimately have applications in humans. There is, however, much more to learn from the animal kingdom before clinical applications are found.

Part 3. Learning and Memory in Birds.

Although birds have, on occasion, been given honorary mammalian status (Thompson, Berger and Madden IV, 1983), they are included in a separate section here because of their obvious importance in relation to the rest of the thesis.

i) Imprinting.

The various forms of learning in birds have become very popular models of learning because birds are very quick learners, particularly at visual tasks. Many young birds are particularly good at learning the visual and auditory characteristics of the mother, during a sensitive learning phase, called imprinting. In this type of learning, information about the identity of the newly hatched chick's mother is encoded rapidly and initiates very strong

approach behaviour. Many biochemical and morphological changes have been found to be associated with both visual and auditory imprinting (Horn, 1985; Scheich, 1987; McCabe and Horn, 1988). Imprinting tends to occur in precocial species that are capable of running or walking soon after hatching and is a mechanism for gaining the maximum amount of protection from the mother hen (Hess, 1973; Bateson, 1974a). It was adopted as a model for studying learning because it was biologically relevant and took place very quickly in the young chick.

Biochemical changes such as increased incorporation of uracil (Bateson, Rose and Horn, 1973) and lysine (Horn, Rose and Bateson, 1973) were recorded in the day-old chick after imprinting on either a rotating red box or a stuffed jungle fowl. An autoradiographic study of uracil incorporation indicated that incorporation was at its highest after training in the medial hyperstriatum ventrale (Horn, McCabe and Bateson, 1979). Similar regions were also highlighted using the 2-deoxy glucose labelling technique after imprinting (Kohsaka et al, 1979).

The cholinergic system was implicated in memory retention for the imprinting task by an increase in cholinergic receptor binding in trained chicks (Bradley and Horn, 1981), while the noradrenergic neurotoxin, DSP4 (an agent that depletes noradrenalin in the forebrain by about 65%) severely impaired training on the red box but not on the junglefowl, implying that memories for the two different stimuli require different neuronal mechanisms (Davies, Horn and McCabe, 1985). Furthermore, the preference score for the junglefowl was correlated with levels of plasma testosterone, while the preference score for the red box was not, giving further indications that different mechanisms were being used for encoding the different visual stimuli (Bolhuis, McCabe and Horn, 1986).

Memory for a similar imprinting task, in which the chicks were presented with a large, moving red sphere, was disrupted by intracranial injection of glutamate, ouabain and cycloheximide (Gibbs and Lecanuet, 1981). Also, N-methyl-D-aspartate (NMDA) receptors have been implicated in imprinting since training caused a 59% increase in receptor binding in the left IMHV but not the right (McCabe and Horn, 1988). However, spontaneous electrophysiological activity in the IMHV decreases with training but increases in the hyperstriatum accessorium (Payne and Horn, 1982). This negative correlation between approach activity and spontaneous neuronal firing in the left intermediate medial hyperstriatum ventrale (IMHV) was found for those chicks trained on the red box but not for those trained on the stuffed junglefowl (Payne and Horn, 1984).

A series of lesion studies were performed by the Cambridge research group. Firstly, bilateral lesions placed in the IMHV both before training (McCabe, Horn and Bateson, 1981) and after training (McCabe, et al 1982) reduced the approach preference of the chicks to chance levels. However, serial unilateral lesions of the IMHV produced more surprising results. When the left or right IMHV was lesioned alone, the chicks displayed recall for the training. However, after the second lesions were made, when the contralateral IMHV was removed, those chicks that had the left IMHV removed last were amnesic while those that had the right IMHV removed last retained an approach preference. The results implied that there were two memory systems used for imprinting and that a permanent store was formed in the left IMHV while an impermanent store was formed in the right IMHV that transferred the memory to some other area when the left was absent. When bilateral lesions were placed in the IMHV, 26 hr after training, the chicks continued to display an approach preference, indicating that by this time the memory had filtered from the right IMHV and was completely stored in some other area. This putative permanent store became called S' (Cipolla-Neto, Horn and McCabe, 1982).

When sequential unilateral lesions were placed in the left and right IMHV before training, both groups preferred the training stimulus, indicating that the learning could be acquired by either hemisphere. If a second lesion was made in the opposite IMHV, both groups had chance approach scores. Interpretation of this result was somewhat vague, but Horn suggested that it indicated there was some interaction between left and right IMHV such that storage of S' required the presence of the left IMHV (Horn, McCabe and Cipollaneto, 1983).

Auditory imprinting has also been used as a model of memory. Chicks are placed in a Y-shaped maze in which one arm contains a hidden stuffed hen and the other is empty. A speaker placed at the end of each arm plays a sound, usually an uninterrupted tone. In one arm the tone is similar to that played previously to the chicks in their home pen, while the other speaker plays a novel tone. Chicks that have become imprinted on the familiar tone will be more likely to choose the correct arm (Maier and Scheich, 1983). Using the 2-deoxy glucose mapping technique three areas of the brain were identified as important for auditory imprinting. These areas are the hyperstriatum accessorium and dorsale (HAD); the lateral neostriatum and hyperstriatum ventrale (LNH) and the medial neostriatum (MNH) (Scheich, 1987).

ii) Song learning.

Another type of learning that has been used as a model of memory in birds, is song learning in canaries and finches. This type of learning has also been called imprinting, although auditory feed back is also required for complete acquisition of song behaviour (Nottebohm, 1977). Song is produced by the syrinx, a structure in the neck that is

innervated by the left and right hypoglossus nerves. Section of the left hypoglossus nerve leads to deficits in vocalisation, while section of the right hypoglossus does not have such a profound effect on song (Nottebohm, 1972; reviewed in Nottebohm, 1984). If, however, the left hypoglossus is cut before Spring, the right hemisphere will assume dominance for song control. Unilateral lesions in the Canary neostriatum and rostral hyperstriatum have no effect on song behaviour but lesions in the caudal hyperstriatum ventrale (HVC, recently renamed the Higher Vocal Center) cause a detriment to song behaviour. Also, by tracing the connections to this area by nerve degeneration, area X of the parolfactory lobe and a round nucleus in the archistriatum (RA) were implicated in song control (Nottebohm, Stokes and Leonardo, 1976).

Large sex differences in the morphology of the canary brain have also been identified. Female canaries have a less well developed area X compared to males. In zebra finches the female area X is completely absent but still apparent in the males. The size differences of nuclei in the male and female were correlated with singing behaviour (Nottebohm and Arnold, 1976). The sex differences in brain structure are controlled by the sex hormone, testosterone, since growth of HVC and RA can be induced in female canaries by gonadectomy followed by injection of testosterone (Nottebohm, 1980). The amount of testosterone in males undergoes seasonal changes and these changes were correlated with seasonal changes in the size of the testes, size of RA and HVC, and with the size of song repertoire (Nottebohm, Kasparian and Pandazis, 1981). It was hypothesised that the seasonal fluctuations in volume of the brain nuclei associated with vocal behaviour were related to increases followed by reductions in the number of synapses in those regions, facilitating greater connectivity between neurons and forming new networks (Nottebohm, 1981). The putative changes in connectivity were hypothesised to take place in successive stages of complexity, to account for the successive stages in song

development, from sub-song, through plastic song to full, or stable song. Continued neuronal development was suggested to account for the increase in repertoire and complexity of song behaviour from season to season (Nottebohm, Nottebohm and Crane, 1986). Golgi studies characterised the hormone sensitive type IV neurons of the RA in female canaries. During hormone-induced song development, new synapses were added throughout the dendritic tree, suggesting that this was the cause of increased volume of the nuclei (Canady et al, 1988).

The question that begs to be asked of the results concerning sex differences in the brains of songbirds is, "Do the differences found in the brains of male and female canaries really relate to a difference in learned information?" It is normally implied that the larger male HVC nucleus is related to a greater amount of stored information concerning its song repertoire. However, the song the male produces can be thought of as a language. If this is the case it would only have meaning for the female bird if she shared the same knowledge as the male. Thus, both male and female birds must store common information concerning birdsong. If this is the case then the sex differences between male and female brains should be considered to be due to the way in which stored information is utilised rather than simply the amount of information stored. The male must transform stored information about his song into complex motor behaviour, while the female must not only be receptive but, for the male to be successful, the female must recognise his song pattern against a background of other auditory inputs, by comparing the new song input against information that was previously stored. Granted, this is a simplistic argument, the male does acquire new information about song since his repertoire increases annually. However, some caution should be exercised in interpreting the results concerning the sex differences between the brains of male and female songbirds.

iii) One-trial passive avoidance learning.

The work on song learning to date has been both unique and informative. However, like imprinting, the findings may be limited to a highly specialised adaptation found in only a few species of birds and do not necessarily reflect changes taking place after a more general form of learning. In the light of this drawback a number of associative learning tasks have been developed for birds, particularly the one-trial passive avoidance task in young chicks. The chick pecks at an aversive flavoured chrome bead, usually dipped in the bitter tasting liquid, methylanthranilate (MeA), causing a disgust response and the acquisition of avoidance behaviour (Lee-Teng and Sherman, 1966; Cherkin, 1969). The control group is usually comprised of chicks that are allowed to peck a similar chrome bead dipped in water rather than MeA. The training and control groups are often referred to as M and W birds, respectively. The passive avoidance paradigm has been used extensively by a number of workers to record many neurobiological correspondents of memory. Also, a "weak" training task was developed with the chrome bead, in which MeA was mixed with water to reduce its aversive qualities. This task was used in an attempt to measure pharmacological facilitation of memory (Cherkin, 1971) with some success. Retrograde enhancement of memory for a "weak" passive avoidance task was reported after mild flurothyl treatment (Cherkin, Meinecke and Gorman, 1975). However, the problem in attempting to measure such a facilitation is that it may be impossible to dissociate learning improvements from increases in attention and performance. Other problems in measuring drug-induced facilitation of cognitive tasks will be explored in chapter 5.

An elaboration of the chrome bead passive avoidance task is the colour discrimination paradigm, in which two different coloured beads are presented to the chick, one of which

is used as the training stimulus by pairing it with MeA, whereas the other acts as a discriminative, or unpaired, stimulus (Gibbs and Ng, 1977). The advantage of a two stimulus discrimination paradigm in studying the effects of pharmacological agents on memory is that amnesia can be dissociated from other pharmacological effects such as changes in motor behaviour.

By using a chrome bead passive avoidance paradigm very similar to that of Cherkin, transient increases in muscarinic and cholinergic receptor binding by the ligand, quinuclidinyl benzilate were found in the brains of trained chicks, implicating the cholinergic system in passive avoidance learning (Rose, Gibbs and Hambley, 1980). An increase in the synthesis and amount of microtubular protein tubulin, persisted for 24 hours after training but was reduced to control levels by 48 hours after training (Mileusnic, Rose and Tillson, 1980). Further work using this task in combination with 2-deoxy glucose mapping highlighted three areas of the day-old chick forebrain, associated with training (Kossut and Rose, 1984). The areas were the intermediate medial hyperstriatum ventrale (IMHV), the lobus parolfactorius (LPO) and the paleostriatum augmentatum (PA). Furthermore, the increase in labelling was greater in the left hemisphere compared to the right (Rose and Csillag, 1985). Many changes were found in the incorporation of fucose into glycoproteins following training, which will be reviewed in part 5 of this chapter.

Protein kinases have also been implicated in memory for passive avoidance training. Phosphorylation of a 52 KDa protein was observed in synaptic plasma membranes isolated from the forebrain of trained chicks, 30 min following training (Ali, Bullock and Rose, 1988a). Intracranial injection of the protein kinase inhibitors, polymixin B and melittin, produced amnesia for the task which developed over a long period of time,

implicating that protein kinases are involved in the formation of long term, but not short term memory (Ali, Bullock and Rose, 1988b).

Training also induced dramatic increases in spontaneous "bursting" of high frequency neuronal firing in the IMHV up to 12 hours after training (Mason and Rose, 1987). The increases were abolished by immediate, post-training subconvulsive transcranial electroshock, a technique that causes amnesia when the shock is administered very soon after training. The "bursting" was not abolished by delayed subconvulsive electroshock, which did not cause amnesia (Mason and Rose, 1988).

Morphological phenomena, possibly related to synaptic remodeling, were found after analysis of electronmicroscope data from the IMHV, 24 hours after training. A number of different parameters were compared between left and right IMHV's of M and W chicks. No difference between left and right presynaptic bouton volume density was found in the W chicks but was 22% greater in the left IMHV compared to the right IMHV of the M chicks. The W chicks had 12% more synaptic vesicles per unit volume of neuropil in the right IMHV compared to the left. However, the reverse difference was found between the left and right IMHV of the M chicks. The mean length of post synaptic thickening was greater, by 12%, in the right IMHV, compared to the left, of W chicks. This difference was not observed in the M chicks. Finally, and most surprisingly, the left IMHV had 61% more vesicles per synapse compared to the right IMHV in M chicks (Stewart et al, 1984).

A follow-up study in the LPO also found a number of training-induced changes in morphology. A 59% increase in the numerical density of synapses was found in the left and right LPO of M trained birds compared to W trained. This was accompanied by a 10% greater synaptic thickening in the right LPO compared to the left in W chicks, which

was reversed in the M trained birds. There was no difference detected in the volume density of presynaptic boutons or mean bouton volume between M and W groups or between hemispheres in similarly trained groups. However, the numerical density of synaptic vesicles and number of vesicles per bouton was 50% greater in the left LPO of M chicks when compared to that of W chicks. No differences were observed in the mean synaptic curvature, mean length of post synaptic thickening, the numerical density of synapses or the volume density of presynaptic boutons. However, the mean bouton volume was greater in the left LPO of M chicks compared to that of W chicks. Finally, the numerical density of synaptic vesicles per bouton volume and the number of vesicles per presynaptic bouton were 15% greater in the right LPO compared to the left, of W chicks. This difference was not found in the M birds (Stewart, Csillag and Rose, 1987).

The subcellular changes observed in electronmicroscope data have been accompanied by cellular changes measured at the light microscope level. Training-induced increases in dendritic spine density were found in both the left and right IMHV, with an asymmetry favouring the left. There were also increases in spine head diameter accompanied by decreases in spine stem length (Patel and Stewart, 1988). When chicks were given transcranial sub-convulsive electroshock, 5 minutes after training, those that still remembered the task had a greater spine density than those that were amnesic, directly implicating that spine increases have some function in memory formation (Patel, Rose and Stewart, 1988).

More recent studies, using electrolytic lesions of the chick forebrain, found that bilateral lesions of the IMHV before training on a coloured bead passive avoidance task caused amnesia (Davies, Taylor and Johnson, 1988), indicating the importance of the IMHV in acquisition of passive avoidance learning. Further work, in which unilateral lesions were

placed in either the left or right IMHV of chicks, before training, indicated that the left IMHV was needed for acquisition of a similar task while the right was not. Bilateral lesions to the IMHV, 1 and 6 hrs after training did not result in amnesia, suggesting that a second area (or areas) takes up the role of long term storage of memory for the passive avoidance task, soon after training (Patterson, Gilbert and Rose, 1990).

A continuation of this study, using the chrome bead task and involving combination lesions of both the IMHV and LPO, was carried out recently. Bilateral LPO lesions made before training did not give rise to amnesia, however, similar lesions performed after training did; implicating this area as a secondary memory store. When pretraining LPO lesions were followed in the same chicks by post-training IMHV lesions, this again resulted in amnesia (Gilbert, Patterson and Rose, 1990). It has been suggested that these results indicate the existence of a secondary storage area similar to S', postulated by Horn (Cipolla-Neto, Horn and McCabe, 1982) but since S' was demonstrated to rely on the intact right IMHV during imprinting, while passive avoidance learning does not appear to require the right IMHV if the left is intact, a similar mechanism in both types of learning is unlikely. However, the recent lesion studies of passive avoidance learning do indicate that the IMHV is needed for acquisition of learning and that some type of transfer of information between left IMHV and LPO must take place.

iv) Pharmacological studies of the stages of memory in birds.

The serial colour-discrimination passive avoidance paradigm has been used in conjunction with various pharmacological treatments to define three sequentially dependent phases of memory formation in the young chick. The paradigm employs the use of two different coloured stimuli, presented to the chick in sequence after it has been

trained to avoid one stimulus by pairing it with MeA. Initially, short-term and long-term phases of memory were identified using ouabain and the protein synthesis inhibitor, cycloheximide. A link between short-term and long-term memory was suggested to involve a sodium pump and the transport of amino acids, initiated by nerve impulse activity and culminating in protein synthesis (Gibbs et al, 1973).

The idea of phases of memory, each requiring different molecular processes, has also been established in other animals. The notion of short- and long-term memory in mice is well documented (Quartermain and McEwen, 1970; Squire and Barondes, 1974). Also a three-phase model of memory has been suggested to interpret amnesia by transient hypoxia in rats (Frieder and Allweis, 1978).

Pretraining treatment with ouabain resulted in decay of retention in young chicks, 10 min after training, while cycloheximide gave rise to amnesia 30 min or more after training (Gibbs and Barnett, 1976). Following further work, short-term memory (STM) was defined as lasting up to 10 min after training and was inhibited by pretraining intracranial administration of lithium chloride or potassium chloride but not ouabain (Gibbs and Ng, 1976). The labile intermediate-term memory (ITM) was found to last between 10 min and 30 min after training and could be disrupted by ouabain. Therefore the intermediate phase was sodium pump dependent. Since the long term phase of memory (LTM) was only inhibited by protein synthesis inhibitors it was proposed that LTM was protein synthesis dependent.

It was postulated that hyperpolarisation due to potassium conductance was the mechanism underlying short term memory while hyperpolarisation due to sodium pump activity was the mechanism underlying the ITM phase. The mechanism for STM was postulated to involve post-tetanic hyperpolarisation (Gibbs, Gibbs and Ng, 1978).

Disruption of STM implied consequent disruption of ITM and LTM, indicating that the three phases of memory were sequentially dependent; information retained during one stage somehow being gradually transcribed from one phase to the next (Gibbs and Ng, 1977).

Work on trained chicks, given no pharmacological treatment, identified two points of temporarily reduced retention, 15 min and 55 min after training. This result was interpreted as indicating the change over from STM to ITM and from ITM to LTM. The second retention "dip" was shifted to 70 min after training if the chicks were housed and trained in isolation instead of in pairs, implying that isolation stress somehow altered the physiological constraints that modulated memory formation (Gibbs and Ng, 1979).

It also became clear that the duration of the phases of memory could be manipulated pharmacologically. The length of STM and ITM was extended, while the time at which LTM was susceptible to cycloheximide was altered, by diphenylhydantoin, an antiepileptic drug (Gibbs and Ng, 1984a). Similar results were obtained using corticosterone and adrenocorticotrophic hormone₁₋₂₄ (Gibbs and Ng, 1984b) and testosterone (Gibbs, Ng and Andrew, 1986), suggesting hormonal modulation of memory.

Roberts (1987) called into question much of the three phase model data presented by Gibbs and Ng over the previous decade, particularly the data implying transient retention dips. Roberts suggested that the data of Gibbs and Ng were less variable than would be expected by chance, assuming that trials with different chicks are independent. Roberts noted that significance tests used by Gibbs and Ng rely on the assumption of independence. However, chicks were tested in pairs, resulting in the possibility that the

outcome of a retention test on one chick may be dependent on the outcome of the other. Roberts went further to retest data as pairs of chicks, assuming a maximum negative dependence within pairs (i.e. that every second chick tested, responded in the opposite way to that of the first chick in the pair) and found that there was still less variance than expected by chance.

Gibbs and Ng (Ng and Gibbs, 1987) responded by conceding that there was less than expected variance in their data. However, they ruled out the possibility of experimenter bias (either deliberate or unconscious) or data manipulation, as explanations for the result. It was noted in their paper that data collected in their lab by students using a similar task, was more variable, indicating that the variance of the data may reduce with the training experience of the experimenter. Thus it appears that the experimenter may influence the outcome of training simply by being a "better" trainer. Indeed, with experience, it is possible for the experimenter to train one chick in a pair without allowing the second to observe this, by presenting the training stimulus in the visual field of one chick only. If the experimenter can maintain this technique successfully for a significant number of pairs of birds, then the training of one chick in a pair could be considered as "independent".

There has been no clear conclusion to this controversy. Gibbs and Ng concluded that less-than-expected variance may be inherent in the passive avoidance procedure, but that this should not bring into question their proposed three-phase model of memory retention. The three phase model is accepted as being a realistic proposition by other workers and the data presented by Gibbs and Ng has, to some extent, been verified (Patterson et al, 1986; Patterson et al, 1988). Permanent amnesia was induced by pretraining injections of glutamate, ouabain and anisomycin. The amnesia developed by anisomycin was evident 60-90 min after training, ouabain induced amnesia 10-20 min

after training and glutamate induced amnesia 5 min after training. Thus Patterson identified similar time periods as Gibbs and Ng for STM and ITM. However, the work by Patterson using anisomycin to disrupt LTM was in conflict with Gibbs and Ng since the amnesia did not fully develop until 75 min after training (Patterson et al, 1988) while Gibbs and Ng claimed that anisomycin developed amnesia 50-60 min after training (Gibbs and Ng, 1977, p.124). This discrepancy may have been due to the fact that Patterson housed chicks individually while Gibbs and Ng housed chicks in pairs. As mentioned earlier, Gibbs and Ng found that the onset of long term memory was delayed by isolation, since the second retention dip in untreated chicks was delayed to 70 min after training in isolation (Gibbs and Ng, 1979). The time course of Patterson's anisomycin experiment fits well with a 70 min delay to the onset of LTM. Thus, isolation may be responsible for the discrepancy between the results of Patterson et al and Gibbs and Ng.

v) Other learning paradigms using birds.

Other associative tasks have been developed with birds. The classic Skinner box was adapted for pigeons by installing two or three illuminated pecking keys. Classical conditioning of pigeons was found to be at least as good as that of rats (Brown and Jenkins, 1968). Go/no-go discrimination and position learning paradigms have been studied more recently using key peck responses of pigeons (Macphail and Reilly, 1985). Lesions of the hyperstriatum ventrale disrupt reversal learning and the acquisition and maintenance of autoshaping in pigeons (Reilly, 1987) as well as classical conditioning (Reilly, 1988) in this paradigm. Lesions of the hyperstriatum dorsale and hyperstriatum accessorium disrupted colour discrimination in a similar paradigm (Shimizu and Hodos, 1989). A key peck task in day-old chicks has also been developed. Chicks will peck at a

key in order to receive heat reinforcement and can learn colour and pattern discrimination (McDougall, Zolman and Mattingly, 1987). Older chicks are also capable of reversal learning in this task (Benowitz and Lee-Teng, 1973).

Interesting results have also been obtained by studying food hoarding and recovery behaviour in the Clarke's Nutcracker and Marsh Tit (Shettleworth, 1983). The task requires an excellent spatial memory as it involves remembering the precise location of nuts in many hiding places (possibly thousands in the natural setting). Lesion of the hippocampus in Black-capped chickadees, after placing food, caused amnesia for the location of the food (Sherry and Vaccarino, 1989). This is one of the few cases in which the hippocampus of birds has been implicated to have a similar role in memory to that of the hippocampus in rats.

The variety of brain research performed using birds is so great that even within this subgroup of animals a synthesis of results is to say the least, difficult. The general fact that has emerged from recent research is that between the huge variety of species and learning paradigms studied, similar forebrain nuclei are continually implicated. In many cases the hyperstriatum ventrale (or substructures within this general area) has become of prime importance, along with some basal nuclei such as the parolfactory lobe and the paleostriatum, in the study of bird memory. There is, however, no reason to suggest that a common mechanism for learning will eventually be found in all bird species, or even in similar species for different learning paradigms. This is ridiculous! Each species has different biological requirements and will therefore have different adaptations to suit those requirements. The laws of variety will be as true of adaptations within the bird's brain as they are of the bird's plumage. It is also very likely that different brain mechanisms are utilised in different types of learning, even within the same species.

Part 4. The Ethology of Learning in the young Chick.

In using animals in the laboratory their behaviour when alive tends to be neglected in the pursuit of more refined techniques for analysing their body tissues when dead. In the light of this, and the fact that the following thesis tends towards the analysis of observations of whole, living animals, rather than dead subdivisions of those animals, there follows a consideration of the behaviour and natural history of the chick.

i) Natural History.

The domestic chick, *Gallus gallus domesticus*, is most probably descended from the wild Burmese red junglefowl and appears to have retained most of the behavioural characteristics of its ancestor (Collias and Collias, 1967). The young chick has good colour vision, its spectral sensitivity is similar to that of the human but is less sensitive to the red and blue parts of the spectrum when light adapted (Armington and Thiede, 1956). The chick eye has more cones than humans; this is probably related to the great importance of colour vision in its normal behaviour. Unlike primates, chicks need no prior exposure to light for colour discrimination to take place (Fischer, 1975). Again, this adaption may be related to the importance of the chick's visual system early in life. There is some evidence of innate bimodal colour preferences, since chicks are more likely to approach colours at the far ends of the spectrum, such as violet and orange, and less likely to approach colours in the green region (Hess, 1956). It was also confirmed that chicks were less likely to approach a 2-dimensional flashing pattern when the pattern was green, compared to other colours such as white, blue, yellow and red (Kovach, 1971). However, differences in light intensity of coloured stimuli make little difference to approach preferences (Fischer and Davis, 1981).

Chicks are small and easily housed in large numbers. They are also relatively cheap (no pun intended) and are highly precocious, being capable of independent exploratory behaviour and learning, less than 12 hr after hatching. Young chicks also have only a minimal blood-brain barrier and a soft, unossified skull, making it very easy to introduce substances into the brain via the systemic or intracranial route. However, it should be noted that for pharmacological studies, the intracranial route of administration is preferred. This guarantees the delivery of an accurate dose to the forebrain and the experimenter need not be too concerned with the effects of body metabolism on the drug administered.

ii) Passive avoidance learning.

The basic one-trial avoidance paradigm of learning in the young chicken, which utilises spontaneous pecking behaviour, has remained practically unchanged since it was first used in the study of memory by Cherkin (1969). This learning model has become popular because it has several advantages. The passive avoidance paradigm is based on spontaneous behaviour, observed in an open field setting (Morgan, 1896), so the chick is highly prepared to acquire the appropriate behaviour (and does so in one trial). Thus it is implied that the acquisition of avoidance behaviour is adaptively very useful. Furthermore, the appropriate learned response is an adaptive behaviour that does not require the acquisition of unnatural movements. Secondly, since the time taken for acquisition is discrete, requiring only one trial, the paradigm is most useful for studying the biological consequences of learning; time course studies of biochemical cascades, electrophysiological and morphological changes become possible, whereas this is not the case for the more traditional multi-trial learning tasks. Thirdly, learning is relatively

permanent, so the study of long term changes within the brain is possible.

However, there are also problems associated with using the passive avoidance response in young chicks as a model of learning. Because the model relies on the suppression of a response rather than the gradual increase of a predetermined response, the experimenter can only record an "all-or-none" score for each subject in the experiment. This means that statistical analysis of behavioural data is non-parametric, since the behavioural data can only be expressed as a frequency within the population. In other words, in order to perform a statistical analysis, the number of individuals displaying retention has to be used to represent the amount of retention. Is this necessarily a good way to measure memory? It may not necessarily be the case that memory is an all-or-none phenomenon in this sense. Cherkin conducted research suggesting that memory was additive and that the strength of a memory trace was related to the strength of reinforcer. He suggested that the memory was expressed once the strength of the memory trace exceeded a given threshold (Cherkin, 1972). It should not be assumed that the frequency at which a given response is expressed in a population reflects a quantitative measure of memory. What is needed is some other, more quantitative measure of memory.

One solution to this problem is to measure other behavioural parameters such as the "peck latency," that is, the time between the chick orientating on the bead and pecking at it. Also the experimenter can count the number of pecks at a bead during a fixed period of time. This approach was used by Cherkin in his attempt to measure memory facilitation by flurothyl (Cherkin, Meinecke and Garman, 1975). It is notable that quantitative measures such as the number of pecks during a fixed period have been reported to be recorded in some papers (Patterson, Rose and Bradley, 1989; Bradley and Galal, 1988; Patterson et al, 1988) but no analysis of the data was made (or if it was, did not appear in the paper). The reason for this may be that data from this type of measure tends to have non-normal

distribution, disallowing most parametric tests. It is, however, possible to analyse "peck latency" data by using Survival analysis, a test designed for highly skewed data. This will be described in more detail in chapter 2.

Although the paradigm has many advantages for the neurobiological study of memory, behaviourally, the passive avoidance paradigm represents a series of compromises. Firstly the chicks are placed in the pens in pairs rather than alone, to reduce the amount of stress behaviour. When alone, the young chick "peeps" continually in order that the mother hen, or the other siblings, can relocate it. Solitary chicks may also adopt a crouching or freezing posture, a defensive behaviour designed to hide the exposed chick from predators. These behaviours are obviously undesirable as they would interfere with training and testing. It has also been shown that isolation has some effect on the time course of memory, apparently extending the period of intermediate term memory (Gibbs and Ng, 1979; De Vaus, Gibbs and Ng, 1980) and may also have an effect on neuronal activity, measured by the 2-Deoxy glucose mapping technique, in areas associated with the biochemical correlates of memory (Muller and Scheich, 1986).

To overcome the problems of isolation behaviour the chicks are housed in pairs but this leads to other bothersome problems when training. The two birds constantly interact within the pen, causing difficulties when training and testing, since the response of one chick may be unduly effected by the attentions of the second bird. Although the chicks are now acting as a pair the experimenter must still consider them separately. He or she must select one bird at a time for any training or testing procedure. Interactions between chicks during testing must be ignored by the experimenter, in spite of the fact that they may have introduced unwanted variation in the data. There is little evidence that chicks acquire a response by empathic learning and the difficulties of training chicks in pairs are

more desirable than those of keeping chicks in isolation. With practice the experimenter can acquire training techniques that largely avoid interference by the second chick, such as presenting the stimulus within the visual field of only one chick.

Since pecking, or rather, the withdrawal of pecking, is assumed to indicate recall in the passive avoidance paradigm, it is important to note what other external stimuli elicit pecking behaviour, in order to eliminate confounding stimuli during recall tests. One such problem is that of peck facilitation, a behaviour that has been observed in young chicks (Tolman, 1964). If one chick observes another peck at a bead it may be more likely to do so itself; again this could complicate any learned behaviour that is displayed by the chicks. Peck facilitation has not, to my knowledge, been proven to override a learned avoidance response, however, I have often noted that if one chick avoids a bead because of neophobia (this happens frequently at the first presentation of a new stimulus) it is likely that the second chick will also avoid, if it has first observed its companion's behaviour (some times this happens even after the second chick has already pecked once at the bead). This may account for high rejection rates, due to untrained animals, in some cases.

A further problem with the paradigm is that the chicks must not be allowed to observe too much activity in the rest of the lab. The presence of the experimenter may be interpreted as a predator by the chick, eliciting fear responses such as crouching and freezing (Jones, Duncan and Hughes, 1981; Suarez and Gallup, 1982). A convenient way of achieving this, and to maintain the correct ambient temperature in the training pen (28-30 °C), is to place a 25 W red light bulb above each pen, while keeping the rest of the lab in darkness. This ensures that the chicks are both warm and can not observe activity in the lab.

The behaviour of young chicks may also be influenced by certain noises. In many cases of passive avoidance training the use of pen tapping was employed to encourage "slow" chicks to peck at the bead during training and the recall test. It has been reported that tapping sounds will elicit feeding behaviour in young chicks (Tolman, 1967) and that the tapping stimulus has its maximum effect at about 120 taps per minute. It is possible that pen tapping mimics the maternal food call, which is a staccato series of short pulses at a rate of 5-7 per second (Sherry, 1977). Although this procedure may be of use in encouraging slow chicks to peck during training, it should not be used during recall test since chicks that recall the training experience may be persuaded to continue pecking at the stimulus and thus be recorded as amnesic.

Apart from stimuli that may directly affect the pecking behaviour of chicks, there are other factors that may add to the behavioural variability of the birds. The time of day that chicks are trained and tested has some effect on the outcome of retention tests (Radford, Ng and Armstrong, 1981). Discrimination learning was most accurate when training took place at 1200 hr while a high degree of generalisation was present when training took place at 0800 hr. When training was performed at 2000 hr there was a low success rate because the chick's general activity was low. Another factor that effects the variability of behavioural experiments is atmospheric pressure (Bateson, 1974b). When the atmospheric pressure was low during day 12 of incubation the chicks were more active than normal after hatching. Groups of chicks may also exhibit different feeding and approach strategies (Gebotys, White and Macdonald, 1984), although these strategies may develop with pecking order and should have little effect on animals removed from the main brood. There is also a possibility that behavioural variation may come about in an experiment due to the differences in sex ratio. Female chicks tend to hatch earlier than males, which may add a bias to experiments, particularly if chicks are only taken from the

earlier part of the hatch (Davies and Payne, 1982). Thus it is always good practice to record the sex of chicks by observing the wing feathers (Hann, 1966).

There is also some question as to what is a good control for the methylantranilate training procedure. There is some evidence that the chick is attracted to peck at the chrome bead because it has similar visual properties to water, and that the chick is prepared to peck at objects with these visual properties in order to find a source of water. In fact, mercury, which has a reflective quality similar to the chrome bead, will act as a super stimulus, initiating a higher approach response than water (Hess, 1956; Rheingold and Hess, 1957). Chicks also show a tendency to peck at droplets or bubbles of water (Wood-Gush, 1955). These observations suggest that the chrome bead acts as a super stimulus, eliciting a greater peck response than other stimuli.

Since the MeA causes the training bead to be wet, it seems reasonable to present a water-dipped bead to the control chicks, thus yielding two groups of birds for biochemical, morphological or electrophysiological analysis; the first group trained to avoid the chrome bead (M) and the second being untrained (W). The ethological evidence suggests then, that the W trained birds may also be learning that the chrome bead represents a source of water. This view is further supported by the results in chapter 4 of this thesis, which suggest that coloured or chrome beads represent potential sources of food or water to the chick. If the W bead is acting as a positive reinforcer, it could be that the W chicks actually learn to peck more at the chrome bead. Thus the neurobiological differences previously found between M and W trained chicks may be due to a comparison between two different types of learning, rather than between a learning and a control group. To overcome this problem a third group should also be observed, in which the chicks have been allowed to peck at a dry chrome bead, or that have had no training experience at all.

Alternatively, biochemical comparisons could be made between chicks that are amnesic after M training (usually a small proportion of chicks, between 5 and 10%, continue pecking at the chrome bead during the recall test, inspite of having expressed a disgust response at training). The size of this group can be manipulated by training with dilute versions of MeA, similar to the technique used by Cherkin (1972). Alternatively, a control group can be generated by using transcranial sub convulsive shock. The timing of the shock after training is critical and will determine the percentage of chicks that are amnesic after training. The drawback in using this technique is that it is not known how the electroshock causes amnesia, however it was used successfully in a recent morphological study (Patel, Rose and Stewart, 1988). More recent biochemical experiments in this lab have incorporated a third, untrained group as a control, yielding results that indicated differences in the amount of c-fos expression between M, W and untrained chicks, the W chicks showing higher levels of expression than the untrained group (Anokhin et al, 1990).

Part 5. Glycoproteins and Memory.

There follows a short review of research on brain glycoproteins and their putative role in memory and learning. The review is by no means extensive and has a strong bias towards the fucosylated glycoproteins, a subgroup of this large family of molecules, which have become of great interest in the study of memory.

i) Structure and synthesis.

The glycoproteins are a large group of macromolecules that can be subdivided into secretory and membrane-bound. For the purpose of this study I shall only deal with the

membrane-bound subgroup. Glycoproteins are proteins with one or more carbohydrate group attached to them. The carbohydrate groups may range from simple to highly complex, consisting of a single residue, di-, tri-, oligo-saccharide; or a highly branched heterosaccharide chain. The predominant sugars in such chains are N-acetylneuraminic acid (NANA), mannose, galactose, N-acetylglucosamine, fucose and N-acetylgalactosamine.

The brain contains high proportions of glycoproteins, approximately 80% of which are to be found in membranous material and are insoluble. A study of the concentration of sugars incorporated into glycoproteins in rat synaptic plasma membranes found 21.4 nmoles/mg of fucose, 33.3 nmoles/mg of galactose and 41.1 nmoles/mg of sialic acid (Gombos et al, 1972)

Commonly the prosthetic groups are bound to the polypeptide chain covalently and lack a serially repeating unit. Beyond these two features the glycoproteins have little in common. Their prosthetic groups vary widely in number, composition and size, as does the composition of the protein moiety. It is for this reason that there is a huge variety of glycoproteins with a multitude of functions ranging from enzymes and receptors or cell-cell recognition and adhesion, to membrane and myelin structure (Irwin, 1974). However, the function of many glycoproteins still remains unexplained.

It is now believed that proteins are glycosylated in the golgi body before being incorporated into the plasma membrane. Synthesis of the protein moiety is carried out as normal while the synthesis of the oligosaccharide is pre-assembled in a carrier lipid, dolichol pyrophosphate. Each carbohydrate residue is added sequentially by substrate specific transferase enzymes, the oligosaccharide is then linked N-glycosidically to the asparagine residue of the polypeptide. Synthesis is completed by the final addition of an

end terminal sugar such as L-fucose. It appears that each stage of the process must be completed before the next one begins, thus glycosylation may be inhibited when only one stage of synthesis is prevented from taking place.

ii) Fucosylated glycoproteins.

Fucose has become of particular interest to neurobiologists studying the function of glycoproteins for a number of reasons. Exogenous fucose is specifically incorporated into glycoproteins in an unchanged form, so radioactively labelled fucose can be used as a specific glycoprotein marker (Margolis and Margolis, 1972; Quarles and Brady, 1971). Also, fucose becomes concentrated in the synaptic terminals and has a relatively fast turnover (Zatz and Barondes, 1970), implicating that fucosylated glycoproteins may have a role in neuronal information processing (see section iv).

There are two known metabolic pathways leading to the incorporation of fucose as the end terminal of glycoproteins (figure 1.1). The first pathway mobilises exogenous and recycled fucose; however in the absence of exogenous fucose, glucose can be metabolised to GDP-fucose (Ishihara et al, 1968). After the systemic injection of large amounts of labelled fucose it has been found that the excess is not metabolised significantly but is excreted by the kidneys in its original form (Coffey, Miller and Sellinger, 1964).

It has been suggested that the significance of a fucose terminal on a glycoprotein is to stabilise the metabolic reactivity of the polysaccharide chain, preventing further addition to the chain and forestalling its breakdown (Margolis and Margolis, 1977). Again, this implicates a role for fucosylglycoproteins in the consolidation of synapses, perhaps by

Figure 1.1; Metabolic pathways for the incorporation of fucose into glycoproteins.

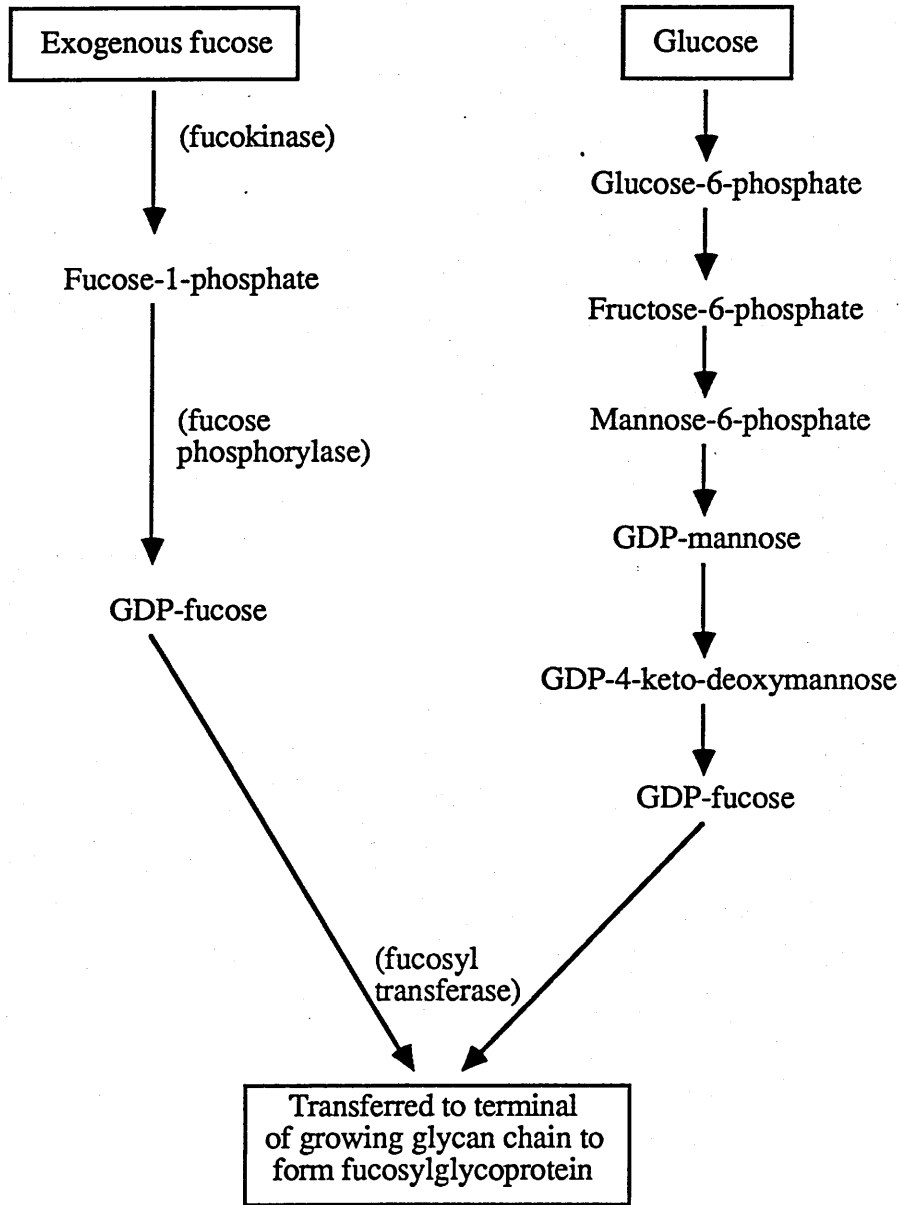


Figure 1.1; The two metabolic pathways of fucose incorporation. Either exogenous and recycled fucose can be utilised, or in the absence of fucose, glucose can be converted to GDP-fucose.

making permanent the changes that have taken place at the synapse after learning.

The importance of fucosylglycoproteins in brain function is illustrated by fucosidosis, a disease in which breakdown of the fucose-galactose bond is impossible because of a lack of the enzyme α -fucosidase, leading to a build up of fucosylglycoproteins. The disease is characterised by severe mental retardation with progressive neural degeneration, leading to death before the age of five (Brunngraber, 1972; Patel, Watanabe and Zeman; 1972).

iii) Inhibitors of glycoprotein synthesis.

Most of the inhibitors of protein glycosylation act on some part of the lipid-saccharide pathway, the metabolic process in which the oligosaccharide is sequentially built onto a lipid carrier and is then transferred to a polypeptide, forming the complete glycoprotein. There are basically two groups of substances that will inhibit this process. The first are antibiotics such as tunicamycin, amphomycin, bacitramycin and diumycin. Of these, tunicamycin is the most widely used. It is a lipophilic analogue of UDP-N-acetylglucosamine and acts by inhibiting the first step in the lipid-saccharide pathway. Because tunicamycin acts so early in the lipid-saccharide pathway it is not a very specific inhibitor. It has also been shown to partially inhibit the incorporation of leucine into protein, even after HPLC purification (Elbein, 1981).

Antibiotics tend to interfere with early stages of the formation of lipid-linked oligosaccharides. Sugar analogues, such as 2-deoxy-D-glucose, 2-deoxy-D-fluoro-D-glucose, 2-deoxy-2-fluoro-D-mannose, glucosamine, fluoromannose and fluoroglucose, interfere with later stages in this process. Most of the sugar analogues inhibit glycosylation by being converted to GDP-deoxyglucose, which interferes with the formation of the lipid-linked oligosaccharide before it is transferred to the growing

polypeptide chain. It is possible that this comes about by GDP-glucose consuming the available dolichol-phosphate, thus preventing glycosylation from taking place (Schwartz and Datema, 1980).

A more unusual inhibitor of glycosylation is 2-Deoxy-D-galactose (2-Dgal), an analogue of fucose (or 6-deoxy-galactose). 2-Deoxy-galactose competes with galactose for incorporation into the oligosaccharide moiety of glycoproteins. Once incorporated it prevents glycosidic linkage with fucose to form the end terminal (Buchsel et al, 1980). 2-Dgal is a unique drug since it only inhibits the completion of fucosylated glycoproteins, thus its action is highly specific. In brain it is mostly metabolised to 2-deoxy-D-galactose-1-phosphate (96% of total metabolite) by the enzymes of the Leloir pathway (Starling and Keppler, 1977a; 1977b).

The amnestic property of 2-Deoxy galactose was demonstrated by Jork et al (1986). When 2-Dgal was injected intraventricularly into rat brains it caused decreased fucosylation in the hippocampus in a dose and time dependent way. This decrease was accompanied by impaired retention of a foot-shock motivated brightness discrimination task.

iv) Glycoproteins and information processing.

The glycoproteins have been implicated as molecules concerned with adaptation within the nervous system for many years; this is only partly due to experimental evidence. For a long time glycoproteins were selected as putative candidates for "information molecules" because they are such a large group of molecules, representing one of the primary constituents of the central nervous system (Di Benedetta, 1974). They are

morphologically localised, located on the outer surface of the plasma membrane and because of their rather peculiar metabolism there are several stages at which they can diversify. For instance, the sugar types and sequence within each heterosaccharide chain, the length of each chain and number and position of chains in the saccharide moiety are all variable factors in the construction of the glycoprotein.

The "information molecule" hypothesis was formulated after experimental evidence implicated glycoproteins in memory (Irwin, 1974). Fortunately, the philosophy behind the study of memory has since changed and few workers entertain the concept of individual molecules encoding a memory. It is now widely accepted that macromolecular changes must occur within the nervous system in order for information storage, however those changes can not be considered to be exclusively responsible for the storage of memory. It is more likely that cellular biochemistry consequent with significant behavioural changes (such as learning) facilitates the necessary, sufficient and exclusive cell biological correspondents of memory formation (Rose, 1981).

There is now much evidence implicating that fucosylglycoproteins have a role in memory formation. Radioactively labelled fucose has been used in a number of different paradigms to demonstrate behaviourally correlated changes in fucose incorporation. Routtenberg and workers found increased [^3H]-fucose incorporation into the caudate putamen after foot-shock motivated active avoidance training, in rats. The changes in incorporation were not found in the hippocampus or cerebellum (Routtenberg et al, 1974). The increases in fucose incorporation were further found to be restricted to three glycoproteins (Morgan and Routtenberg, 1979). Increased [^3H]-fucose incorporation has more recently been found in the hippocampus after training on a brightness discrimination task (Popov et al, 1980). Increased incorporation was found in the area dentata soon after training followed by the CA3 between 7 and 9 hours after training. There were also two

peaks of fucose incorporation in the CA1, 3 and 9 hours after training. These findings were followed by the discovery of increased activity of fucokinase and decreased activity of fucosyltransferase, two enzymes involved in utilising L-fucose, in hippocampus after brightness discrimination learning (Popov et al, 1983).

After passive avoidance training in day-old chicks, fucose incorporation was found to increase, 30 min after training. This change lasted for at least 24 hours after training (Sukumar, Rose and Burgoyne, 1980). It was also found that incorporation of fucose was highest in the membrane fraction after intracerebral injection (Burgoyne and Rose, 1980). Also, the increase in fucokinase activity was unusually high in the right forebrain base by 14% over control, one hour after passive avoidance training (Lossner and Rose, 1983).

An elegant experiment using sub-convulsive transcranial electroshock to cause amnesia demonstrated that the increased fucose incorporation following passive avoidance training, in the day-old chick, was not due to concomitant factors of the training technique, such as stress or increased motor behaviour. Incorporation of [^3H]-fucose was increased in the forebrain base and anterior forebrain roof after passive avoidance training but was abolished by immediate, amnesia-causing, electroshock. If the shock was delayed, however, no amnesia was evident but an increase in fucose incorporation was still detected (Rose and Harding, 1984).

It has also been demonstrated that fucose incorporation into completed polypeptides takes place soon after learning, in vitro. The increased incorporation was greatest in tissue taken from the right forebrain base after training (McCabe and Rose, 1985). The protein synthesis inhibitor, cycloheximide, inhibited [^3H]-fucose incorporation only by 60%,

indicating that some protein must have been synthesised before inhibition and stored for fucosylation (Mc Cabe and Rose, 1987).

Curiously, some results have suggested that injection of L-fucose facilitates performance of some learning tasks. L-fucose injected either intraperitoneally or intracranially, improved retention in rats for both shuttle-box and shock-motivated brightness-discrimination tasks. Similar injections of the D-isomer of fucose did not yield this result (Wetzel et al, 1980). It was suggested that the endogenous supply of fucose normally acted as rate limiting, when this constraint was lifted by the injection of excess L-fucose, the fucosylation of glycoproteins could be maximized, somehow bringing about an ability to learn more efficiently. Similar results were obtained for the brightness discrimination task by injecting D-galactosamine and D-glucosamine before training (Popov, 1985). It is however, difficult to separate true facilitation of learning from other factors that may be caused by the administration of drugs, such as enhanced performance and altered awareness.

Recently sulphated sugars have been demonstrated to cause amnesia and it is thought that they do so by interfering with fucosylation of glycoproteins. Heparin and other sulphated polysaccharides caused a decrease in fucosyltransferase activity (Jork and Lossner, 1985). Also, the fucose analogue, 2-Deoxy galactose, caused amnesia for the foot-shock motivated brightness discrimination task. The amnesia was correlated with a decrease in hippocampal fucosylation (Jork, Grecksch and Matthies, 1986). This result was confirmed in the day-old chick. Bilateral intracranial injection of 20 μmol 2-Deoxy galactose inhibited [^3H]-fucose incorporation into acid insoluble material by 26%, within 1 hr of injection. A similar dose of the drug also induced amnesia for the chrome bead passive avoidance task but injection of similar concentrations of galactose, glucose or 2-

D-glucose did not. The amnesia induced by 2-Dgal was abolished by a simultaneous injection of 40 μ mol concentrations of galactose, but not fucose, implying that the 2-Dgal was competing with galactose for the same incorporation site. No amnesia was detected when 2-Dgal was administered 4 hr before, or 3 hr after training. The greatest percentage amnesia was obtained when 2-Dgal was injected between 1.5 hr before and 1.5 hr after training, implying that there is a 2 to 3 hr time window in which the drug is effective (Rose and Jork, 1987).

It remains to be demonstrated that 2-Dgal will cause amnesia for a discrimination task and whether the drug causes amnesia in a state dependent way, by becoming an internal cue, necessary for recall of the learned experience. It is also of interest to determine whether the drug will cause amnesia in any other learning paradigm. The following thesis aims to meet these goals and to discuss further the implications of the amnesia induced by 2-Dgal.

CHAPTER 2. DEVELOPING AND TESTING A SERIAL COLOUR-DISCRIMINATION PASSIVE AVOIDANCE TASK.

INTRODUCTION

Day-old chicks peck spontaneously at small brightly coloured objects. This behaviour has been employed in the now classic one-trial passive avoidance paradigm, in which the experimental chicks are allowed to peck at a chrome bead dipped in the bitter tasting liquid, methylantranilate, while control chicks are encouraged to peck a similar bead dipped in water (Cherkin, 1969). The consequent learned avoidance is robust, being evident in 80% to 90% of trained individuals and many neurobiological changes have been found to take place after training (Rose, 1989). The paradigm is of great value in studying the biochemical correlates of the training experience since learning takes place after only one trial, allowing the measurement of biochemical changes at discrete times after learning. To study the effect of pharmacology on memory, however, this simple paradigm is not quite adequate.

A major shortcoming of the chrome bead paradigm is that only one stimulus is used to train and test the chicks. It is assumed that withdrawal of pecking behaviour indicates that learning has taken place. However, this behaviour may be ambiguous in a pharmacological study since a drug treatment may cause withdrawal of the peck response as well as altering memory. An amnestic drug may, for instance, also cause drowsiness or altered motor activity, leading to withdrawal of pecking behaviour. Using the chrome bead paradigm, this response would be interpreted as evidence of recall. Thus a discriminative stimulus must be introduced into the paradigm; recall would then be

indicated by avoidance of the training (or paired) stimulus accompanied by continued pecking at the second (unpaired) stimulus. This type of paradigm has been employed extensively by Gibbs and Ng (Gibbs and Ng, 1979; Gibbs, Ng and Andrew, 1986; Gibbs, Richdale and Ng, 1987), who trained chicks to avoid a red bead but continue to peck at a similar sized blue bead. In this design the beads were presented one after the other to each chick in turn and the behaviour of the chick was recorded as either "peck" or "avoid". It could be argued that this is not a true discrimination task since the subject does not see the two stimuli simultaneously; this is why the task has been described here as "serial discrimination." Although the two stimuli are never presented simultaneously it should be noted that the time between presentation of the first and second stimulus is minimised by the experimenter.

In our laboratory the chicks were housed under red light in an otherwise dark room, so illuminated stimuli were used in preference to simple coloured beads. The bicolour light emitting diode (LED) was chosen as training stimulus (for a full description see appendix). The LED has the capacity to emit two different colours, determined by the direction of flow of current. It was chosen as a training stimulus since the unpaired stimulus would be identical to the paired stimulus in every respect other than colour. Also, the LED would be very visible in the dimly illuminated training pen. Initially the aim was to design a paradigm similar to that used by Gibbs and Ng (Gibbs and Ng, 1979), in which chicks were trained to avoid a red bead but continue to peck at a blue bead. However, the nature of the bicolour LED dictated that we use red-green discrimination, since red-blue LEDs are not manufactured. The choice of a red-green combination may have introduced some bias into the training procedure since there is some evidence of approach preferences, suggesting that chicks are more attracted to the orange and blue regions of the spectrum and least attracted to the green region (Hess,

1956). There is also evidence that green is less preferred as an imprinting stimulus, compared to yellow, red, blue and white (Kovach, 1971) but this should not create a problem in this paradigm, since the red-green LED is not intended to be an imprinting stimulus.

With the above evidence of approach preferences in mind the following experiment was performed to determine how effectively day old chicks can learn a red-green discrimination and to establish whether one stimulus is more likely to elicit pecking behaviour. The experiment was also designed to test for any order effects due to the serial presentation of the paired and unpaired stimuli.

Experiment 2.1; Testing a serial colour-discrimination passive avoidance paradigm.

METHOD

Day-old chicks of both sexes were placed in metal pens in pairs at 9am. The chicks in each pair were identified by a spot of animal dye, placed on the back of one chick before being introduced to the pen. The floor of each pen was covered with blue lab tissue and scattered with chick starter crumbs. Once introduced into the pen the chicks were allowed an acclimatisation period of at least 1.5 hr. For further details of the housing conditions, see appendix 1.

At the beginning of the experiment each chick was presented in turn with a small white bead (2.5 mm in diameter), to initiate pecking behaviour, and then given two pretraining

trials separated by 15 min intervals. Two LED's had been constructed, one to be used for pretraining and retention trials while the other was used exclusively for the methylantranilate presentation. Each pretraining trial consisted of two 10 sec presentations of the LED (4 mm in diameter), lit either red or green (for design specifications of the training LED see appendix 2). On each occasion the LED was presented for 10 sec, quickly removed and the colour changed, then presented again for a similar length of time. The order of presentation of the two colours was varied from pen to pen. 15 min after the second pretraining trial each chick was presented with the training LED lit either red or green and dipped in methylantranilate (MeA). On pecking at the training stimulus, chicks normally displayed the classic disgust response of head shaking and bill wiping. The training stimulus was held in the chicks visual field for a further 10 sec after pecking, in order to ensure that the chick saw the stimulus both before and after pecking. Those chicks that did not peck during the training trial or did not peck at least once at each LED colour during pretraining were discarded at the end of the experiment (39.5%). The majority of chicks that were discarded would not peck at one of the two colours during the pretraining trials and could not, therefore, be included in a discrimination task.

A retention test followed 4 hr after training, in which the red and green LED was presented to each chick in turn for 10 sec. Again the order of presentation of red and green was varied from pen to pen. The response of each chick to each stimulus was recorded as either "peck" or "avoid". The data were analysed using the Chi square test of independence with Yates' correction for continuity, on each possible pair of conditions and expressed as the percentage of chicks avoiding each stimulus (% avoiding). Also the number of chicks that learned a true discrimination, that is, those chicks that avoided the paired stimulus during the retention test but continued to peck at the unpaired stimulus,

was counted and expressed as % discriminating.

RESULTS

A comparison between the number of chicks that avoided the stimulus paired with MeA (86.5%) and those that avoided the unpaired stimulus (65.4%) yielded a significant difference ($\chi^2=5.27$, $p\leq 0.05$). 33% of chicks learned to discriminate, that is, they avoided the paired stimulus but continued to peck at the unpaired stimulus. Table 2.1 gives a summary of the data broken down by training colour and stimulus presentation order. The results are shown graphically in figure 2.1.

Table 2.1: Number of chicks avoiding the red and green LED after training.

Training colour	Presentation order	n	N avoiding red	N avoiding green	N learning to discriminate
Red	red-green	13	12 (92%)	6 (46%)	6 (46%)
Red	green-red	12	11 (92%)	10 (83%)	1 (8%)
Green	red-green	14	7 (50%)	11 (79%)	4 (29%)
Green	green-red	13	11 (85%)	11 (85%)	4 (31%)

A comparison of the two stimulus presentation orders yielded no significant difference in the number of chicks avoiding the paired stimulus ($\chi^2=0.00$) or the unpaired stimulus ($\chi^2=0.00$). Also the colour of the training stimulus had no significant effect on the number of chicks avoiding the paired or unpaired stimuli ($\chi^2=0.5$ and $\chi^2=0.01$ resp.)

Figure 2.1; A counterbalanced test of red-green discrimination learning in chicks.

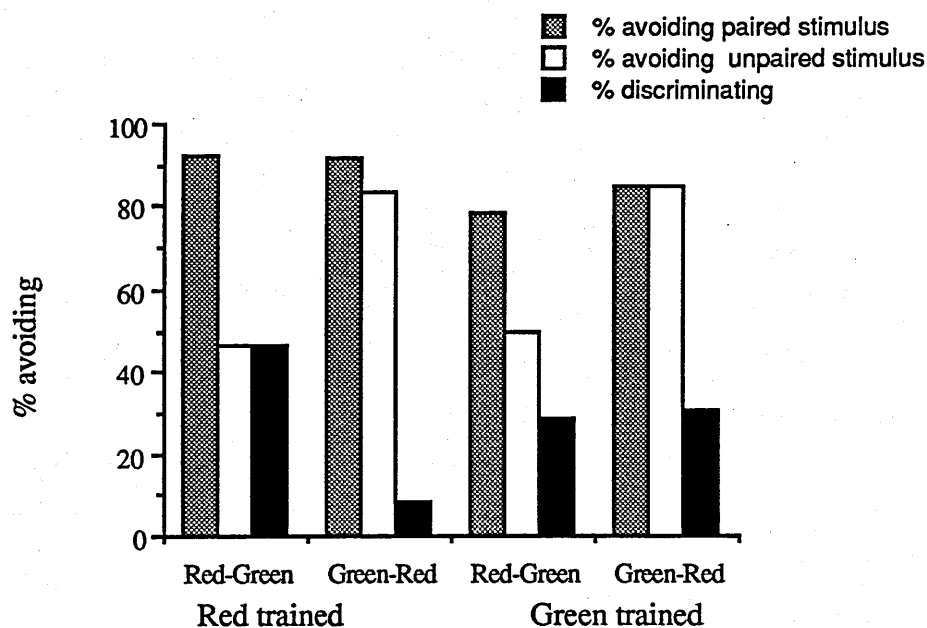


Figure 2.1; The number of chicks avoiding the red and green LED after training with methylantranilate. More chicks avoided the stimulus paired with MeA than the unpaired stimulus ($p \leq 0.05$). There was no significant difference in the number of chicks avoiding either stimulus, due to order of presentation of stimuli during the recall test. The number of chicks discriminating (i.e. avoiding the paired stimulus and pecking at the unpaired stimulus) is also shown.

The number of chicks learning to discriminate (avoid the paired colour but continue to peck at the unpaired colour) varied. However, when data was grouped by presentation order or by training colour there was no significant difference in the number of chicks discriminating (by stimulus presentation order, $\chi^2=0.50$; by training colour, $\chi^2=0.01$), therefore the apparent variation in the number of chicks discriminating may have been due to an interaction between the colour of the training stimulus and the order of presentation of stimuli during the recall test.

DISCUSSION

The differences between the number of birds avoiding the "paired" and "unpaired" stimuli in both the red trained and green trained groups indicate that chicks are capable of learning the task with both stimuli. There was no effect due to the order of presentation of stimuli during the retention test. Although figure 2.1 shows an apparent variation in % avoiding between the four different conditions, this variation may simply be due to small subject numbers in each condition, since no overall differences due to training colour or presentation order could be found.

The results demonstrate that the training paradigm is robust since learning was evident after training with both LED colours. Therefore it should not be necessary to use a fully counterbalanced design in future experiments, one training colour and one presentation order will provide an adequate design. Because of this the number of different treatment groups needed in any experimental design can be kept to a minimum.

A much greater number of chicks avoided the unpaired stimulus, compared to the results

of Gibbs and Ng (Gibbs and Ng, 1979). This may be because they used red-blue discrimination, not a red-green discrimination as used here. However, the difference in % avoiding the paired and unpaired stimuli (23%) was considered high enough to indicate discrimination learning. The method of presentation of this data is likely to be misleading since the serial presentation of "paired" and "unpaired" stimuli can be considered as related events. So there are four possible outcomes of a serial presentation of two stimuli: avoid "paired" but peck "unpaired" (discrimination); avoid "paired" and avoid "unpaired" (generalisation); peck "paired" and peck "unpaired" (amnesia); peck "paired" but avoid "unpaired" (amnesia or confusion). This point has been made previously in a study on the effect of time of day on discrimination learning (Radford, Ng and Armstrong, 1981). In that study the data was segregated into the four outcomes listed above, however, only two parameters were compared, implying that some data must have been discarded for analysis. The reason for this approach becomes obvious when one contemplates the analysis of data expressed in terms of the four parameters described. Since the data is nominal, one would have to perform repeated χ^2 tests, comparing each treatment group by each behavioural parameter, generating: $4\{(1_i+2_i+3_i+\dots+N_i)-N\}$ comparisons, where i represents an individual comparison and N represents the number of treatment groups in the experiment. Thus the previous experiment would generate 24 individual comparisons. This does not include individual comparisons of behavioural parameters within treatment groups. Obviously this approach is unrealistic; so many individual comparisons would be generated that the data in all but the most simple of experiments would be impossible to interpret.

The number of chicks that did not meet the training criteria was high (39.5%). Two pretraining presentations of the LED were used to maximise the number of chicks reaching the training criterion of pecking at least once at each colour before training. A

number of chicks initially tended to avoid both colours but this behaviour was extinguished, in some individuals, by the second pretraining trial. Most of the chicks were rejected because they would only peck at one of the two colours during the pretraining trials. No pattern to this behaviour was detected, it appeared that some birds had an individual colour preference and would only peck at one colour, but no general colour preference was detected.

To determine whether this behaviour followed a general trend, a second analysis was carried out using the pretraining data from all chicks used (including those rejected). Table 2.2 gives a summary of the results.

No difference between the number of chicks avoiding red and green could be found in either the 1st pretraining trial ($\chi^2=1.25$) or the second ($\chi^2=1.14$). Also, there was no significant difference between the two pretraining trials, in the number of chicks avoiding red ($\chi^2=2.83$) or the number avoiding green ($\chi^2=3.01$). However, both values indicate a trend towards more chicks avoiding both colours in the second pretraining trial. This implies that pecking behaviour would habituate to both stimuli over repeated presentations.

The increased avoidance in the second pretraining trial is undesirable but must be balanced against the fact that if only one pretraining trial was used, more chicks would have to be rejected by the rather strict training criteria (48.8% with only one pretraining trial, but 39.5% with two). It seems counterintuitive that the % avoidance score in the second pretraining trial was higher than the first but that the number of chicks rejected was lower after the second trial. The reason for this is that some birds peck quickly at

both beads initially and then habituate (but still peck at the training bead when it is presented) while other chicks initially display neophobia to one or both colours which may then attenuate to some extent.

Table 2.2: Number of chicks avoiding the pretraining stimuli in each pretraining trial.

Presentation order	n	1st pretraining trial		2nd pretraining trial	
		n avoiding		n avoiding	
		red	green	red	green
Red-Green	44	16 (36%)	16 (36%)	23 (52%)	19 (43%)
Green-Red	42	18 (43%)	10 (24%)	23 (53%)	19 (45%)

The paradigm used in the following experiments only employed one of the four possible combinations of training colour and presentation order, to reduce the number of treatment groups used in pharmacological studies. Green was chosen as the training colour and red-green as the presentation order. This decision was arbitrary, however if the data shown in figure 2.1 is observed it will be noted that under these circumstances the % avoiding and % discriminating scores represent an approximate median of the overall scores.

In the following experiments the "peck latency" (latency of each chick to peck each stimulus) was recorded in order to obtain an interval measure of behaviour. The "peck latency" is defined as the time in seconds between the chick orientating to the stimulus and pecking at it. If a chick has been trained to avoid a specific stimulus, a short peck

latency to that stimulus is assumed to indicate amnesia while a long or maximum peck latency is assumed to indicate recall of the training experience.

The peck latency data can be analysed by computer, using Survival Analysis (SPSSx user's manual, pp. 874-887, 1986) in the SPSSx package held on the Walton Hall VAXcluster mainframe computer. This type of analysis is similar to that suggested recently (Allweis, Chernilovsky and Shultz, 1989) for the analysis of passive avoidance data from rats and mice. Survival analysis is used when the dependent variable represents the time interval between an initial event and a termination event. The termination event may occur outside the maximum time period allowed by the experiment. In the present case the initial event is represented by the orientation of a chick to the LED and the termination event is the chick pecking at the LED. The maximum time period allowed for observation in the following study was 20 seconds; if a chick did not peck within this time period (because it remembered to avoid the LED), it was assigned a 20 second score. Survival analysis treats observations of 20 seconds as censored data, such that the survival time for those observations is not known exactly but is assumed to be at least 20 seconds. A survival score is calculated for each observation by comparing the survival time for that observation with all other observations in the same treatment group. Censored observations are considered to have a survival time greater than the maximum observation time (20 seconds in this case). Survival scores are used in comparisons to determine whether individual treatment groups differ significantly in terms of survival. The D statistic is then calculated from the survival scores using the algorithm of Lee and Desu (Lee and Desu, 1972). D is asymptotically distributed as chi-square with $g-1$ degrees of freedom (where g is the number of treatment groups in the experiment). The larger the D statistic, the more likely it is that the treatment groups come from different survival distributions.

Normally, multiple analysis of variance (MANOVA) would be used to analyse data from behavioural experiments. However, the distribution of data from this experiment is very skewed, since the mean of any treatment group will tend towards 20 seconds, depending on how many individuals avoided the LED during the recall test. A multiple factor analysis is invalid for this data, since MANOVA requires data that is normally distributed. By using survival analysis it is still possible to segregate and compare the data by various factors, such as types of drug treatment, sex of chicks, pen number and so on, in the same way as MANOVA. If, for instance, a drug and control group of chicks have been trained to avoid the green LED and the peck latencies and sex of each bird have been recorded, then the peck latencies of male chicks in the drug group can be compared with those of males in the control group, or alternatively the latencies of all the chicks in the drug group can be compared with those of all the chicks in the control group. Thus all possible comparisons between individual groups can be made using this statistic.

Experiment 2.2. A behavioural dose-response study of 2-Deoxy galactose.

INTRODUCTION

2-Deoxy galactose (2-Dgal) is a fucose analogue that competes with galactose for incorporation into the carbohydrate moiety of glycoproteins, preventing fucosylation. Rose and Jork (1987) found that amnesia developed in chicks trained on the chrome bead passive avoidance task when 5 μ mol 2-Dgal was injected into each forebrain hemisphere,

both before and after training, making a total dose of 20 μmol . The amnesia was not produced by injections of glucose, fucose or 2-Deoxy glucose. Also, the amnesia could be abolished by large doses of galactose but not by other sugars.

In the study by Rose and Jork, the drug was administered in two separate pulses, before and after training. In the following study a dose-response test of 2-Dgal was carried out, followed by an experiment to determine the time course of the effect of 2-Dgal when administered before training only. In order to deliver the equivalent dose of 2-Dgal to that used by Rose and Jork, the dose administered in each injection had to be doubled to 10 μmol in stead of 5 μmol .

The following experiment was designed to determine that different doses of 2-Dgal will cause amnesia in the chrome bead passive avoidance task, while experiment 2.3 tested the amnesic effect of 2-Dgal in the serial colour-discrimination task described in experiment 2.1.

METHOD

The amnestic effect of 20, 30 and 40 μmol of 2-Dgal was compared with 0.9% saline, in a chrome bead passive avoidance task with a similar protocol to that used by Rose and Jork (1987). The drug was made up in a 10 μl volume and injected bilaterally. All injections were performed 45 minutes before training. This time was selected as a matter of convenience since the drug has a time window of about 2 hr in which it is effective (Rose and Jork, 1987). The injections were performed freehand, using a Hamilton syringe fitted with a plastic stopper to ensure that the drug was delivered at a constant depth of 4mm below the skull. This technique delivers the drug to an area of the brain

consistent with the IMHV, but does not necessarily limit the distribution of the drug to a discrete area.

The training protocol for the dose-response experiment comprised two pretraining presentations of a small white bead followed by one presentation of a dry chrome bead, each pretraining trial being separated by 5 min. The training trial followed 10 min after the last pretraining trial and involved a 10 sec presentation of the chrome bead dipped in MeA. A retention test was carried out 3 hr after training and involved a single 10 sec presentation of the dry chrome bead to each chick in turn. The behaviour of each chick during the retention test was recorded as either "peck" or "avoid." Only those chicks that pecked during at least two of the pretraining trials and also pecked the MeA dipped bead, exhibiting a disgust response, were included in the analysis.

RESULTS

Figure 2.2 illustrates the results of the dose-response experiment, while table 2.3 gives a summary of the data. The results indicate that a dose of 20 μmol 2-Dgal caused fewer chicks to avoid the bead, compared to the saline injected group ($\chi^2=4.24$, $p\leq 0.05$). The greater doses of 30 and 40 μmol did not have this effect ($\chi^2=2.95$ and $\chi^2=0.35$ respectively).

It should be noted that χ^2 comparisons between individual drug groups were not significant (20 μmol Vs 30 μmol , $\chi^2=0.00$; 20 μmol Vs 40 μmol , $\chi^2=1.49$; 30 μmol Vs 40 μmol , $\chi^2=0.75$)

Figure 2.2; A behavioural dose-response study of 2-Deoxy galactose.

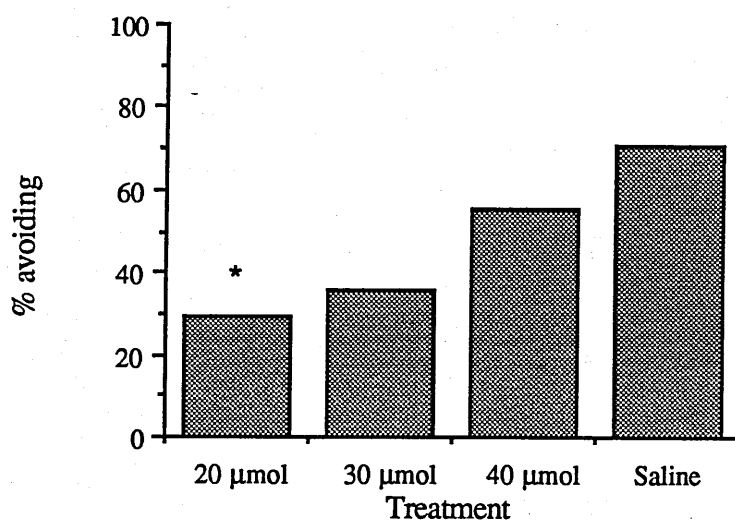


Figure 2.2; A dose-response test of 2-Dgal. The amnestic effect of three different doses of the drug was compared with that of 0.9% saline, using the chrome bead passive avoidance task. Injections were administered 40 min before training and a retention test was performed 3 hr after training. All birds were trained to avoid a chrome bead by pairing it with MeA. The number of chicks avoiding the bead in the retention test was significantly lower in the 20 µmol group compared to the saline group ($p \leq 0.05$), indicating that amnesia was caused by the lower dose of 2-Dgal but not by the 30 or 40 µmol doses.

Table 2.3: Comparison of the effects of different doses of 2-Dgal compared with the effect of 0.9% saline.

Treatment group	n	% avoiding	χ^2 *	p≤
20 μ mol 2-Dgal	17	29.4	4.24	0.05
30 μ mol 2-Dgal	17	35.3	2.95	NS
40 μ mol 2-Dgal	18	55.6	0.33	NS
0.9% saline	17	70.6	-	-

* Comparison with % avoiding in saline group.

DISCUSSION

The results indicate that a dose of 20 μ mol caused amnesia for the chrome bead task. The higher doses of 30 and 40 μ mol did not cause amnesia. There are two possible explanations for this. Firstly the behavioural effects of 2-Dgal could follow a U-shaped curve in a similar fashion to other drugs, such as β -endorphin (Martinez and Rigter, 1980). However, this effect is usually found with drugs that act as receptor agonists, at concentrations that cause receptor fatigue (Martinez and Kesner, 1986). An alternative explanation for the U-shaped curve phenomenon is that different doses of the drug have opposing effects. For example, the effect of noradrenaline on evoked potentials on the rat hippocampus, a system that is mediated by α - and β -adrenergic receptors. Low doses of the drug increased the magnitude of evoked potential by acting as an α -agonist while high doses inhibited the response by acting on β -receptors (Mueller et al, 1982).

The higher doses of 2-Dgal could have caused apparent recall, in this case, by having opposing behavioural effects. The higher dose of the drug could have caused the chicks to become drowsy during the retention test. Since a positive response in this paradigm is expressed by the withdrawal of pecking behaviour, adverse drug effects could be interpreted by the experimenter as recall. This illustrates the value of a two stimulus discrimination paradigm, since drowsy animals would not be expected to peck at either stimulus, while recall would be expressed as withdrawal of pecking at the paired stimulus with continued pecking at the unpaired stimulus.

Experiment 2.3. Testing 2-Deoxy galactose as an amnesic agent in the serial colour-discrimination task.

METHOD

In the following experiment a bilateral intracranial injection of 10 μ mol 2-Dgal was administered in 10 μ l of 0.9% saline, just before pretraining (45 min before training). The control group was injected bilaterally with a similar volume of 0.9% saline. The training procedure described in experiment 2.1 was followed. During the retention and pretraining tests, the red LED was always presented before the green LED, however, during the training trial only the green LED dipped in MeA was presented. Retention tests were performed 1, 2 and 3 hrs after training. The same chicks were used for all three retention tests. Chicks that pecked at only one colour during the pretraining trials or did not peck at the training LED within 10 sec were rejected at the end of the experiment. During each presentation of the LED the "peck latency" was recorded using a hand held stopwatch. The peck latency was defined as the time in seconds between the chick

orientating to the LED and pecking at it. Those chicks that did not peck the LED during the retention test were assigned a peck latency of 10 sec.

All injections were performed "blind" by having a separate person code the identical vials containing drugs and saline. The code was not broken until the experiment was completed. The data were analysed using Survival Analysis (SPSSx held on the Walton Hall VAX mainframe computer) which employs the Lee-Desu D statistic (Lee and Desu, 1972).

RESULTS

Figure 2.3 illustrates results of the time course experiment. The data and statistical analyses are also summarised in table 2.4. There was a significantly shorter mean peck latency to the green LED, in the 2-Dgal injected group compared to the saline group, 2 hr and 3 hr after training ($p=0.043$ and $p=0.009$ respectively). The peck latency to red LED, 2 hr after training, was also significantly shorter in the 2-Dgal injected group ($p=0.008$). However there was no significant difference between the peck latencies of the 2-Dgal and saline group 1 hr after training ($p=0.068$), although the result indicated a trend towards amnesia in the 2-Dgal injected group.

Figure 2.3; Time course of the development of amnesia induced by 2-Deoxy galactose.

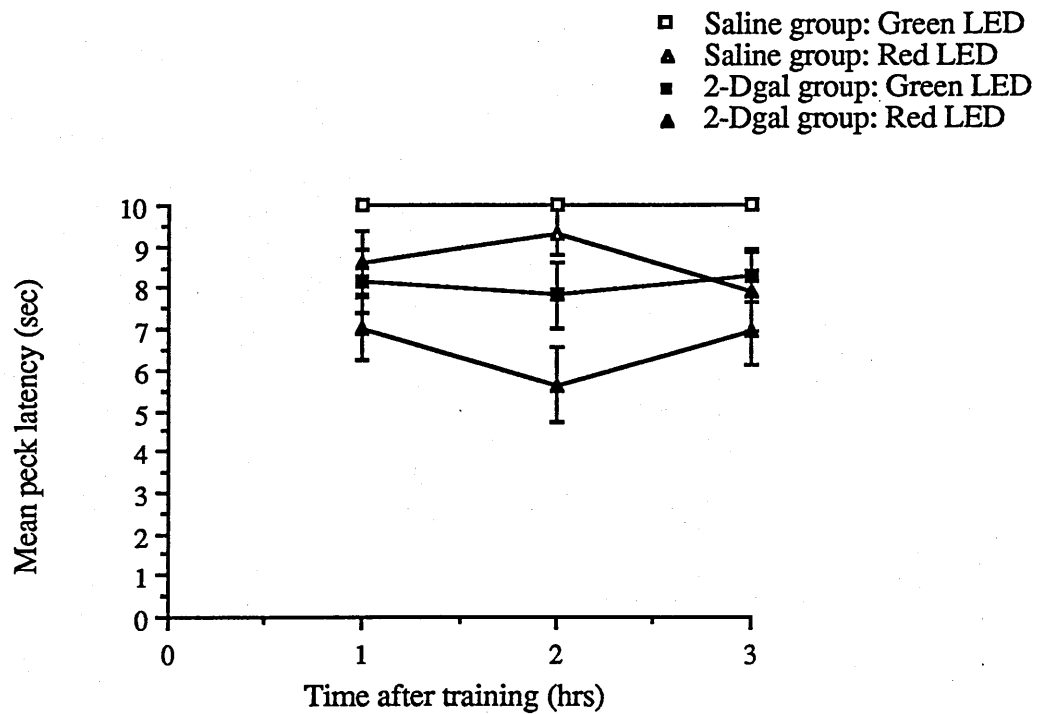


Figure 2.3; Mean peck latency of chicks treated with either saline or 2-Dgal. The peck latency to the green LED was significantly shorter in the 2-Dgal injected group, 2 and 3 hr after training ($p=0.043$ and 0.009 respectively) but not 1 hr after training ($p=0.068$), indicating amnesia at those time points.

Table 2.4; Statistical comparison of mean peck latencies of 2-Dgal and saline injected groups, for both red (unpaired) and green (paired) LEDs.

	n	Retention test					
		1hr		2hr		3hr	
		red	green	red	green	red	green
2-Dgal group	22	7.0	8.1	5.6	7.8	6.9	8.3
saline group	13	8.6	10.0	9.3	10.0	7.9	10.0
D statistic	-	1.34	3.34	7.14	4.41	0.9	6.78
p value	-	0.247	0.068	0.008*	0.043*	0.344	0.009*

*=significant difference

To provide a comparison between the peck latency measure and the previously used % avoiding measure, the % avoiding data have been reproduced in table 2.5. A 3-dimensional χ^2 analysis was carried out on the green LED % avoiding data (Zar, 1984) to investigate interactions between the three factors of Time, Drug and Number Avoiding. This test has the advantage that repeated pair-wise comparisons need not be carried out on the data, so the risk of a type I error is reduced.

Table 2.5; % Avoiding scores of 2-Dgal and saline injected groups.

	n	Retention test					
		1hr		2hr		3hr	
		red	green	red	green	red	green
2-Dgal group	22	54.6	77.3	40.9	72.7	50.0	59.1
saline group	13	69.2	100.0	84.6	100.0	69.2	100.0

The 3-dimensional χ^2 test measures the overall homogeneity of three factors and then test for partial independence between pairs of factors. The overall test for mutual independence of the data described in table 2.5 yielded $\chi^2=14.07$, $p\leq 0.025$, indicating that Drug, Time and Number Avoiding are not all mutually independent. Tests for partial independence indicated that Drug was not independent of the other two factors ($\chi^2=11.07$, $p\leq 0.025$), Number Avoiding was not independent ($\chi^2=17.15$, $p\leq 0.005$) while Time was an independent factor ($\chi^2=1.87$). When the data were combined over Time for a standard two-way analysis it was found that fewer chicks avoided the green LED in the 2-Dgal injected group ($\chi^2=12.7$, $p\leq 0.001$). Thus 2-Dgal caused amnesia for the training task but it can not be stated, with this analysis, that the amnesia changed with time.

DISCUSSION

The results show that amnesia developed slowly in the 2-Dgal injected group, causing a

significantly shorter peck latency 2 hr but not 1 hr after training. There are a number of possible reasons for the delay in the amnesia developed by 2-Dgal. Firstly the effect could have been due to a delayed action of the drug, because it took time to diffuse to its area of greatest effect. This is fairly unlikely since the injection technique introduces the drug directly into the forebrain in an area consistent with the IMHV, there is no need for diffusion across the blood-brain barrier. However, it is quite likely that the drug diffuses throughout the forebrain during the 2 hr period in which it is active.

It is likely that 2-Dgal has its main effect on molecules required for intermediate and long term memory, not short term memory. The effect of the drug may be on a metabolic process that is upstream of the events required for memory formation. This is a more likely explanation since the drug is probably acting to inhibit the gradual formation of glycoproteins some time before their utilisation in memory formation, in the same way that a protein synthesis inhibitor may prevent long term memory formation by prior inhibition of protein synthesis.

Thirdly, the effect may be due to a metabolite of the drug rather than the drug itself. 2-Dgal is metabolised by the Leloir pathway (Starling and Keppler, 1977a) producing 2-Deoxy-D-galactose-1-phosphate as its main product. It is possible, but unlikely, that the delayed amnesia was due to this metabolite of the drug.

There may have been some habituation of pecking due to repeated presentation of the stimuli over a number of retention trials; although one would expect a similar effect in the saline controls. To avoid this problem the following studies employ only one recall test for each chick.

It was noted that a number of chicks pecked the LED stimulus just outside the 10 sec presentation period and had to be scored as avoiding. It appeared that chicks were generally more reluctant to peck the LED compared to the chrome bead. Thus, one further improvement in the paradigm was to increase the presentation time of each stimulus from 10 sec to 20 sec. This helped to increase the number of chicks trained; the 10 second cut-off time may have imposed a ceiling limit on data that was too restrictive, excluding chicks that may have pecked within 20 sec but not within 10 sec.

It should be noted that the alternative analysis following table 2.5 indicated that a time course of slowly developing amnesia after 2-Dgal treatment may be a false conclusion. It is the case that repeated pair-wise comparisons of data, as performed by the Survival analysis test, may increase the chance of making a type I error. Thus the conclusions drawn from the results of this technique should be treated with suspicion. Although the 3-dimensional χ^2 analysis provides a novel and more appropriate alternative to analysing these types of data, the test will not measure differences between individual pairs of data points.

In the following chapter a second paradigm, involving a sickness-conditioned aversion, will be developed and tested. The lateralised function of fucosylglycoproteins in both paradigms will be investigated in chapter 4.

CHAPTER 3. THE ROLE OF GLYCOPROTEINS IN SICKNESS-CONDITIONED AVERSION LEARNING.

INTRODUCTION

Attempts have been made to formulate a unifying theory of learning. However, some learning paradigms have produced results that obstruct those attempts. In traditional learning paradigms, a conditioning stimulus (CS) is paired with an unconditioned stimulus (US), giving rise to a conditioned response (CR). This type of learning is under a number of constraints. The conditioning will normally only be completed after more than one pairing of the CS and US. More importantly, the time between CS and US is critical (Perin, 1943) and must be limited in order that the US does not become associated with a second, undesirable CS. Traditional Pavlovian experiments yield an optimal CS-US interval of between 0.25 and 2 seconds (Bitterman and Schoel, 1970).

One example of a learning paradigm that does not follow the normal rules is taste aversion, or sickness-conditioned aversion learning. The paradigm generally involves allowing an experimental animal to drink two novel coloured and flavoured saccharine solutions. After drinking one of the solutions the subject is made ill by an intraperitoneal injection of a toxin or exposure to a high dose of X-irradiation. On future presentation of the two novel solutions the animal will avoid the solution paired with illness but will continue to drink the unpaired solution (Garcia and Ervin, 1968; Garcia et al, 1968). The learning is highly stimulus specific; when irradiation follows the ingestion of two foods, one novel and the other familiar, an aversion of the novel food is acquired but the animal will continue to eat the familiar food (Revusky and Bedorf, 1967). This paradigm has been used to explain "bait shyness" in wild rats, in which the rats quickly learn to avoid

poisoned bait by sampling small quantities of new food and avoiding that food if it makes them ill.

Sickness-conditioned learning is robust, taking place usually after a single trial, and has been reported in many species (for review see Gaston, 1978a) including rats (Garcia and Ervin, 1968), a number of birds such as pigeons (Westbrook, Clark and Provost, 1980) and domestic chickens (Gaston, 1977), primates (Johnson, Beaton and Hall, 1975) and humans (Bernstein, 1978).

The important difference between sickness-conditioning and classical learning paradigms is that the illness can still be associated with the food previously eaten even if there is a long time delay between the CS and US, for many hours in some cases (Garcia, Ervin and Koelling, 1966). The dilemma faced by psychologists at the time of the discovery of sickness-conditioning was that memory of the novel food must be retained for a substantial period of time before it becomes associated with illness. Thus a memory was being retained without immediate reinforcement.

Previous attempts have been made to explain sickness-conditioning (Revusky, 1977; Garcia et.al., 1985). The stand taken by Garcia is that there are several types of learning and that rules concerning one type can not be applied to another. This is not a new idea, Tolman also suggested that there is more than one type of learning and that the concept of a unifying theory was redundant (Tolman, 1949). However, this approach does not address the problem that still remains: there must be some neural or macromolecular mechanism to retain information for a long period of time before it becomes associated with sickness. It is possible that the molecular processes required to retain a memory are similar for different types of learning. Thus, there is the possibility of a unifying

mechanism for all types of learning, even though different types of learning are expressed at the behavioural level.

Further studies of sickness-conditioning show that rats are specifically prepared to associate illness with previous gustatory stimuli, and are more likely to make this association than between, for instance, illness and visual stimulation (Garcia et al, 1968). An important aspect of this type of learning is that animals tend to be highly prepared to acquire the learning for some sensory modalities but not others. For instance, the rat is more prepared to acquire an aversion to the taste cues provided by a food, rather than the visual properties (Garcia et al, 1968), or auditory cues (Holder, Bermudez-Rattoni and Garcia, 1988). This particular preparedness in the rat is not surprising; the rat is normally nocturnal so it is more prepared to use gustatory and olfactory modalities, rather than visual, in identifying food (Barnett, 1975). A comparison of sickness-conditioning in rats and quail found that the quail was more prepared to associate illness with the visual characteristics of a food rather than with gustatory cues (Wilcoxon, Dragoin and Kral, 1971). Thus, sickness-conditioning is common to many animals but individual species may be prepared to learn the association by different sensory modalities.

In accordance with classical theories of learning, models of the cellular mechanisms of memory have been based on experiments in which animals learn novel responses after the contiguous pairing of CS and US. According to Hebb's (1949) original hypothesis, the contiguous activity of pre- and post-synaptic neurons provides the signal for the consolidation of the synapse between them. This has formed the theoretical framework in which to fit experimental observations of biochemical correspondents of memory formation. If it can be established that sickness-conditioning can still take place with visual information concerning the conditioned stimulus but no gustatory information, it then becomes of considerable interest to ask whether making this brain representation

involves biochemical processes similar to those indicated in more traditional learning paradigms. If, for instance, glycoprotein synthesis occurs during sickness-conditioning, in the period after presentation of the CS but before the US, it would be difficult to account for such a phenomenon by assuming that the signal for the new synthesis resulted from the contiguous excitation of two neural pathways, one stimulated by the CS, the other by the US.

In the following experiments a sickness-conditioned aversion paradigm was developed for day-old chicks, using the green LED as a training stimulus. The paradigm employs the propensity of day-old chicks to peck spontaneously at the dry LED, thus they are not provided with gustatory cues. Furthermore, it was demonstrated that the memory for this task was dependent on the synthesis of fucosylated glycoproteins by administration of 2-Deoxy galactose.

Experiment 3.1; Developing and testing a sickness-conditioned aversion paradigm.

METHOD

A total of 68 day-old chicks were placed in pairs in metal pens in the usual way. They were not, however, provided with chick crumbs or any other food or water. The chicks were allowed to acclimatise for 1.5 hours and were then each presented with a small white bead to initiate pecking activity. Half an hour later they were each given a 10 second presentation of the green LED or a 4 mm diameter chrome bead (as used in the chrome bead passive avoidance task; see Rose and Jork, 1987). Most chicks pecked at the training lure at least once; those that did not (<30% on each day) were withdrawn

from the experiment. Half an hour after "training" with the CS, the birds were injected intraperitoneally either with LiCl (0.1 ml of 1.0 M LiCl made up in 0.9% saline), a dose sufficient to make the chicks noticeably sick for about 1.5 hours, or with a similar volume of 0.9% saline. The intraperitoneal injections were performed "blind" by having the drug containers labelled by an independent experimenter. Training and retention tests were performed by the author while the intraperitoneal injections were performed by Dr David Gilbert after I had left the room. This approach was used since it was quite easy to determine which vial contained the LiCl by observing which chicks became ill after injection. Thus, to perform a truly "blind" investigation, two experimenters were required. Two hours after injection, all birds were provided with water in a petri dish. Preliminary investigations had indicated that this aided the recovery of the LiCl-injected chicks.

Retention tests were performed four hours after training. The chicks were tested by a second presentation of the CS (either the green LED or the chrome bead), again lasting 10 seconds. Each bird was also presented with the stimulus (chrome bead or green LED) that had not been paired with sickness during training. The order in which chrome bead and LED were presented was alternated for each pair of birds to cancel any possible order effects of stimulus presentation. During the retention test the behaviour of each chick was recorded as either "peck" or "avoid," this data was expressed as % avoiding and analysed using the Chi square test of independence with Yates' correction for continuity. For each chick the two stimuli were labelled "paired" and "unpaired" according to which had been paired with the injection of LiCl at training.

RESULTS

Figure 3.1 gives the results from the sickness-conditioned aversion procedure (also summarised in table 3.1). Significantly more of the chicks injected with LiCl avoided the paired stimulus at testing than birds injected with saline (63% vs 20%, respectively, $\chi^2=10.98$, $p\leq 0.001$), whereas there was no difference between the groups in the number avoiding the unpaired stimulus (29% vs 23%, $\chi^2=0.06$). Further χ^2 comparisons revealed no significant difference in the number of LiCl-injected birds avoiding the LED or the chrome bead ($\chi^2=0.16$), nor did it matter which stimulus was used to train the saline-injected chicks ($\chi^2=0.89$). Also, there was no significant order effect, due to the stimulus presentation order at testing, in the lithium injected ($\chi^2=0.73$) or the saline injected groups ($\chi^2=2.17$).

Table 3.1; Summary of data from counterbalanced study of the sickness-conditioned aversion paradigm.

Training stimulus	Treatment	n	Number Avoiding	
			paired stimulus	unpaired stimulus
green LED	Lithium	16	11 (68.8%)	3 (18.8%)
green LED	Saline	15	4 (26.7%)	3 (20.0%)
chrome bead	Lithium	22	13 (59.1%)	8 (36.7%)
chrome bead	Saline	15	2 (13.3%)	4 (26.7%)

Figure 3.1; Testing the sickness-conditioned aversion paradigm.

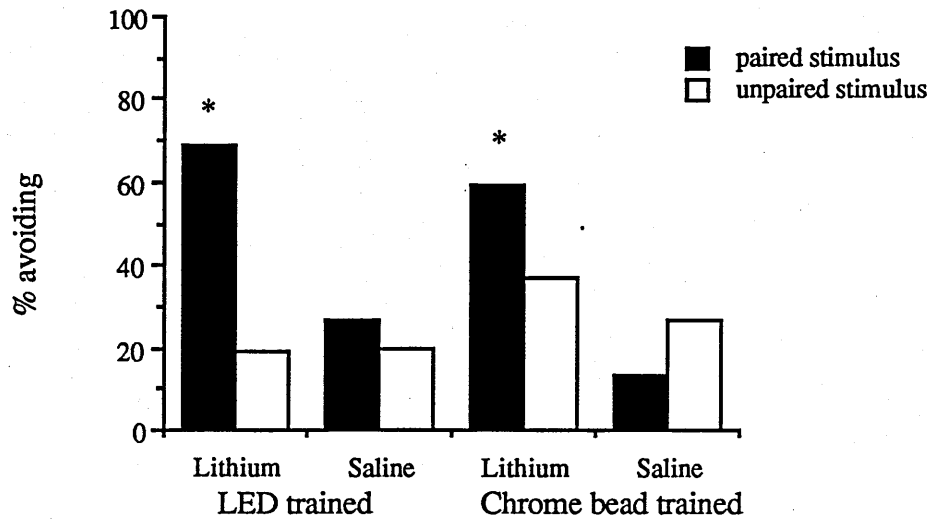


Figure 3.1; Groups were injected i.p. with either lithium chloride or saline after training with the green LED or the chrome bead. A retention test followed 4 hr later and involved the presentation of both the LED and the chrome bead. The order of presentation of the two stimuli was varied. Significantly more chicks in the lithium injected group avoided the paired stimulus during the retention test compared to the saline injected birds ($p \leq 0.001$). There was no significant difference between groups in the number of chicks avoiding the unpaired stimulus.

DISCUSSION

The results indicate that sickness-conditioned aversion learning can take place when either the green LED or the chrome bead is used as the training stimulus, suggesting that sickness-conditioning in chicks must be a phenomenon that is not specific to one stimulus only. The results also indicate that the learning is very strong, being evident in a large proportion of the experimental animals (approximately 60 % avoiding compared to 20 % in the control group). There was no effect due to the order of presentation of the paired and unpaired stimuli. Thus the paradigm is robust and the future experimental design need only employ one of the two training stimuli. This will help to conserve the number of experimental groups needed in future designs.

It is notable in this example of sickness-conditioning that no gustatory cues were provided to the chick by the training stimulus, implying that the chick interprets the LED and chrome bead as sources of food. It also implies that primarily visual information concerning the training stimulus must be stored during the 30 min between CS and US.

The following short experiment was carried out to determine how time dependent the injection of LiCl was for this learning, by delaying the injection for longer periods of time after training.

Experiment 3.2; Investigating the optimum time delay between CS and US of sickness-conditioning.

METHOD

60 day-old chicks were trained as described in experiment 1, by a 10 sec presentation of the green LED. The chicks were separated into three groups and the individuals in each group were injected intraperitoneally with 0.1 ml of either 0.9% saline or 1.0 M LiCl. The three groups were given the intraperitoneal injection 30 min, 60 min and 90 min after presentation of the green LED, respectively. In order to perform the experiment "blind", training and retention tests were carried out by the author, while D.B.Gilbert carried out the intraperitoneal injections in the author's absence.

Retention tests were conducted 4 hr after training and consisted of serial 10 sec presentations of the green LED and chrome bead, to each chick in turn. The order of presentation of the two stimuli was varied from pen to pen. The response of each chick to each stimulus was recorded as "peck" or "avoid" and the data was analysed by χ^2 comparisons.

RESULTS

Scores from saline and LiCl treated groups were compared for each injection time. More chicks injected with lithium 30 min after training, avoided the LED during the recall test, compared to those injected with saline ($p \leq 0.025$). There were no differences in the number of chicks avoiding, between lithium and saline injected groups, in the 60 min and 90 min groups. Thus an acquired aversion to the green LED was evident when lithium

was injected 30 min, but not 60 or 90 min, after presentation of the LED. The results are illustrated in figure 3.2 and summarised in tables 3.2 and 3.3.

Table 3.2: Summary of the data from experiment 3.2, expressed as the % avoiding the green LED and chrome bead.

Treatment group	Injection Time	n	% avoiding LED	% avoiding chrome bead
LiCl	30 min	8	87.5	12.5
Saline	30 min	10	20.0	10.0
LiCl	60 min	14	28.6	7.1
Saline	60 min	8	37.5	0.0
LiCl	90 min	14	50.0	7.1
Saline	90 min	6	33.3	0.0

Table 3.3: Summary of χ^2 comparisons between LiCl and saline injected groups, for each injection time.

Injection Time	LED		Chrome bead	
	χ^2	p \leq	χ^2	p \leq
30 min	5.63	0.025	0.028	NS
60 min	0.02	NS	0.599	NS
90 min	0.04	NS	0.451	NS

Figure 3.2; The effect of varying the time delay between training and injection of lithium chloride, on sickness-conditioning.

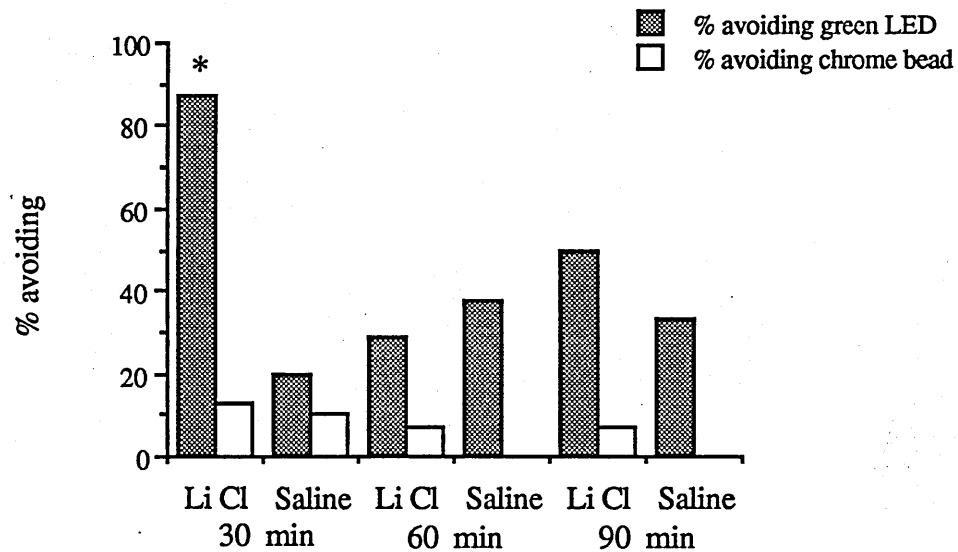


Figure 3.2; Three groups of chicks were allowed to peck the green LED for 10 sec and then injected with either LiCl or saline, 30 min, 60 min or 90 min later. The retention test was carried out 4 hr after training and consisted of serial presentations of the LED and chrome bead. More chicks avoided the green LED in the group injected with lithium 30 min after training, compared to those injected with saline ($p \leq 0.025$), indicating that a conditioned aversion had been acquired by this group. No other comparisons were significant.

It should be noted that pair-wise comparisons between the scores of lithium groups also indicated that learning took place in the 30 min group but not the 60 and 90 min groups (30 min Vs 60 min, $\chi^2 = 4.91$, $p \leq 0.05$; 30 min Vs 90 min, $\chi^2 = 1.69$, NS; 60 min Vs 90 min, $\chi^2 = 0.59$, NS). Also, an overall χ^2 comparison between lithium treated groups gave a result of $\chi^2 = 7.08$, $p \leq 0.05$.

DISCUSSION

At first this result appears to be surprising, since the young chick does not taste or ingest any food when pecking at the bead or LED. But since the sense of smell and taste are relatively less well developed in the chick (King-Smith, 1971) it is likely that it relies much more on vision, which is well developed, to identify food. It may be that in allowing the chick to peck at a bead or green LED we have supplied it with all the necessary information normally required to recognise a food. If this is the case it has important implications for any experimental design involving chicks pecking at beads. In the chrome bead passive avoidance paradigm a water dipped bead is used as the control stimulus and it is assumed that W-trained chicks do not learn anything. The results reported here, however, indicate that the chick is prepared to remember the experience of pecking at a bead, without having been paired with a reinforcer such as MeA or water, for 30 min or more.

Since there is a significant delay between the CS and US in this learning paradigm, information concerning the CS must be remembered without association with the US. Later, when the chick becomes ill, memory of the characteristics of the CS must somehow be retrieved and associated with the US. The question of how this memory is initially stored in this type of learning has been important to psychologists since it was

first discovered. However, no satisfactory neurological explanation has yet been provided. In the context of this thesis it becomes important to determine whether the memory for this task can be disrupted by 2-Deoxy galactose, thus indicating that it requires the synthesis of fucosylglycoproteins, in the same way as for passive avoidance learning.

In the following experiment 2-Dgal was injected intracranially before the chicks were allowed to peck at the training stimulus, in an attempt to abolish memory for the task.

Experiment 3.3; The effect of 2-Deoxy galactose on sickness-conditioned aversion learning.

METHOD

A procedure similar to the one described in experiment 3.1 was used, except that only the green LED was used as a training stimulus. 10 min before training each chick was given a bilateral intracranial injection of either 2-Dgal (10 μ mol in 10 μ l 0.9% saline) or 0.9% saline. The intracranial injections were performed "blind" by having a second experimenter code the vials containing drug and saline. Injections of LiCl were also performed "blind" by D.B. Gilbert, 30 min after training, as described previously.

Each chick was given a retention test, 4 hr after training, by presenting the green LED and the chrome bead for 10 sec each. As in previous experiments, the order of presentation of the green LED and chrome bead was alternated. The behaviour of each chick was recorded as "peck" or "avoid" and the results were expressed as % avoiding. Figure 3.3 is a schematic representation of the protocol used in the experiment.

Figure 3.3; Schematic representation of the protocol of experiment 3.3.

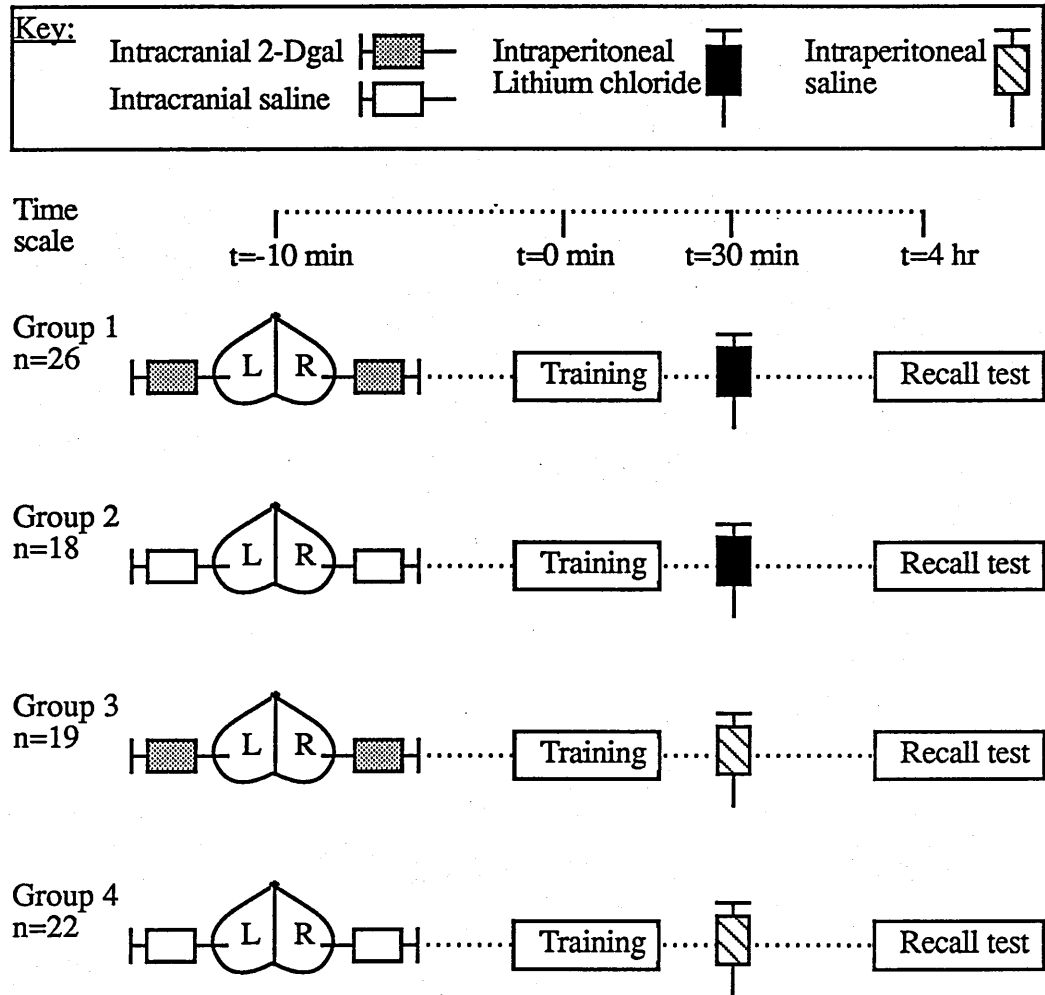


Figure 3.3; Protocol for experiment 3.3. Four groups of chicks were given bilateral intracranial injections of either 2-Dgal or saline. 10 min later they were presented with a green LED for 10 sec. 30 min later those chicks that pecked the LED were given an intraperitoneal injection of either LiCl or saline. A recall test was carried out 4 hr after training, by 10 sec presentations of a chrome bead and green LED

RESULTS

Figure 3.4 illustrates the effect of bilateral intracranial injection of 2-Dgal on sickness-conditioning and summaries of the data and statistical comparisons are provided in tables 3.4 and 3.5. Of the chicks injected i.p. with lithium chloride, those injected i.c. with 2-Dgal avoided the LED less than those injected i.c. with saline ($\chi^2=5.32$; $p\leq 0.05$), indicating that 2-Dgal induced amnesia for the training experience. A comparison between the group injected i.p. with LiCl but i.c. with saline and the groups injected i.p. with saline did not give significant results ($\chi^2=2.21$ and $\chi^2=3.53$ respectively). No group avoided the chrome bead significantly more than any other. However, the individual pair-wise comparisons may be in error since an overall comparison yielded a significant difference ($\chi^2=7.91$, $p\leq 0.05$).

Table 3.4; Summary of the data from experiment 3.3.

i.c. Treatment	i.p. Treatment	n	% avoiding LED	% avoiding chrome bead
2-Dgal	Lithium	26	26.9	7.7
saline	Lithium	18	66.7	16.7
2-Dgal	Saline	19	36.8	10.5
saline	Saline	22	31.8	9.1

Figure 3.4; The effect of pre-training injection of 2-Deoxy galactose on sickness-conditioning.

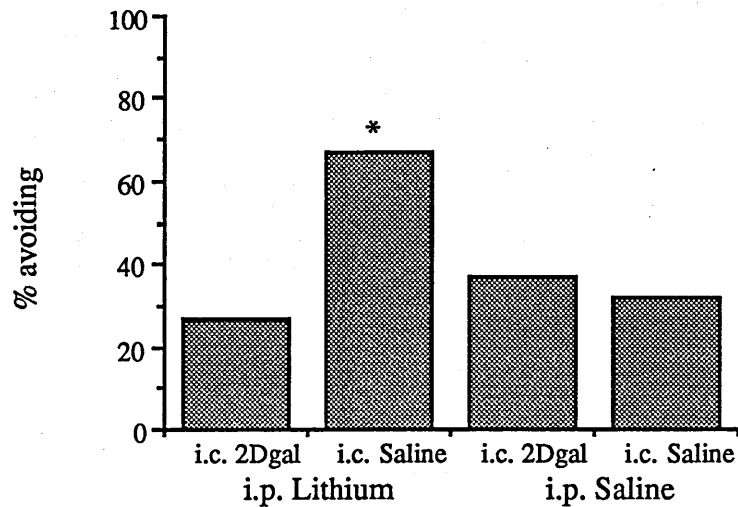


Figure 3.4; Four groups of birds were trained in the way described in figure 3.3. The chicks were injected bilaterally, i.c. with either saline or 2-Dgal, 10 min before a 10 sec presentation of the green LED. 30 min later the chicks were injected with either lithium or saline and given a retention test 4 hr after training. More chicks avoided the LED in the group injected i.c. with saline and i.p. with lithium, compared to the i.c. 2-Dgal group ($p \leq 0.05$).

Table 3.5: Summary of statistical comparisons of % avoiding LED.

Comparison*	χ^2	P
(2-Dgal - Lithium)	5.32	$p \leq 0.05$
(2-Dgal - Saline)	2.21	NS
(saline - Saline)	3.53	NS

*(i.c.injection - i.p.injection), all comparisons are with (i.c. saline - i.p. lithium group).

DISCUSSION

The results indicate that 2-Dgal injected bilaterally, 10 min before training caused a profound amnesia for the learning task. The memory for this task must therefore require the synthesis of fucosylglycoproteins.

The time window of action of 2-Dgal is at least 2 hr long (Rose and Jork, 1987) so it is likely that the drug exerts its metabolic action for a protracted period of time from injection into the CNS. Thus, it is unclear from this experiment, what aspect of the learned aversion is being interfered with by the drug. It could be that the acquisition of visual information (during the training trial) requires the synthesis of glycoproteins, or it may be that this synthesis is only required for the association of that information with sickness, after the injection of lithium. In the following experiment, the injection of 2-Dgal was delayed until after training, in order to address this question.

Experiment 3.4; The effect of a delayed injection of 2-Deoxy galactose on sickness-conditioned aversion learning.

METHOD

A total of 105 chicks were used over a period of 4 days. On each day the chicks were separated into four groups and each given an intracranial injection of 2-Dgal, identical in dose to that of experiment 3.3, 20 min after presentation of the green LED, rather than 10 mins before. The injection of LiCl was still administered 30 min after training with the green LED. The protocol for this experiment is shown schematically in figure 3.5.

RESULTS

As illustrated in Figure 3.6, there was no difference in the number of chicks avoiding the LED, between the two i.p. LiCl groups ($\chi^2=0.98$). This suggests that the delayed injection of 2-Dgal produced no amnesia. A comparison between the two groups injected i.c. with 2-Dgal yielded a significant difference ($\chi^2=5.29$, $p\leq 0.05$). Also, a similar comparison between the two groups injected i.c. with saline was significant ($\chi^2=8.05$, $p\leq 0.01$). An overall χ^2 comparison of the green LED data yielded a significant difference ($\chi^2=17.72$, $p\leq 0.001$). Thus, the results indicate that learning took place in both the groups injected i.p. with LiCl. Summaries of the data and the statistical comparisons are presented in table 3.6 and 3.7 respectively.

Although the data are not shown in figure 3.6, it should be noted that there were no significant differences between groups, in the percentage of chicks avoiding the chrome bead (see table 3.7).

Figure 3.5; Schematic representation of the protocol of experiment 3.4.

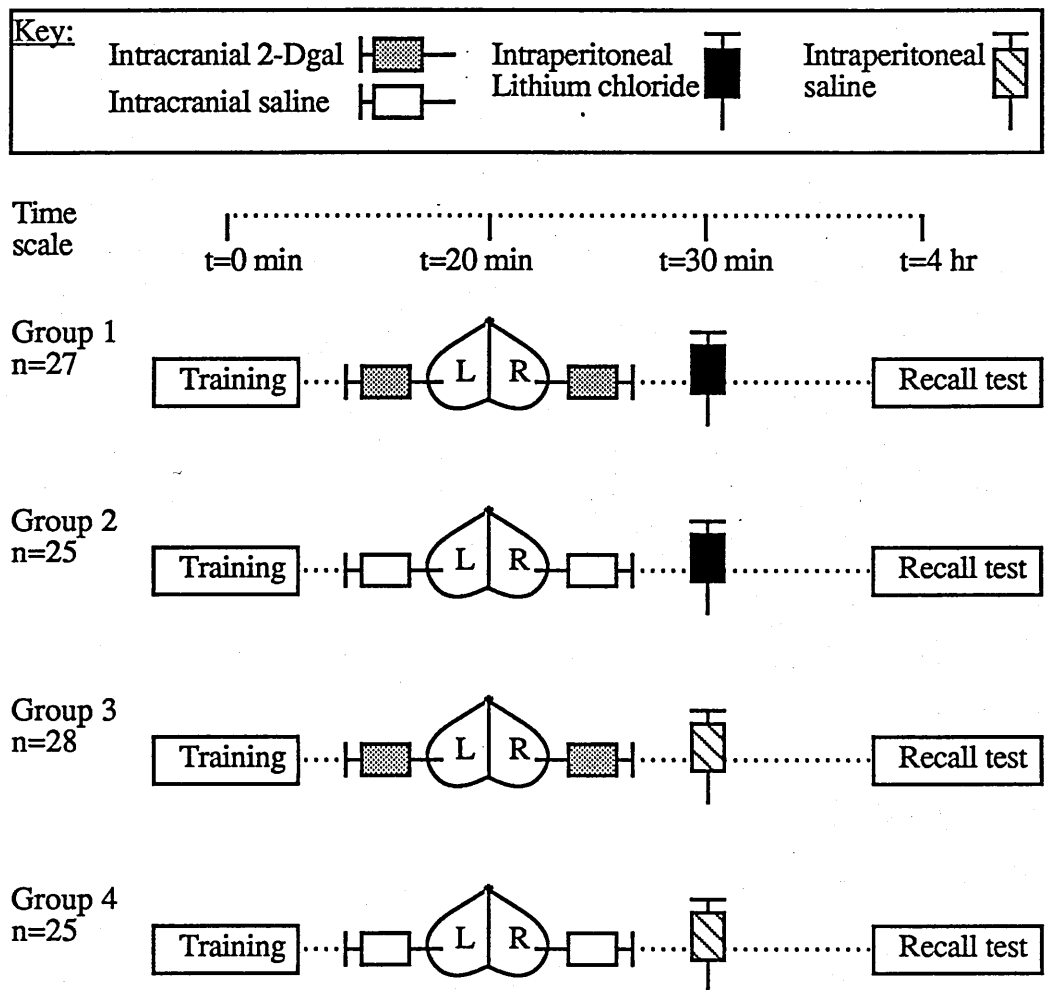


Figure 3.5; Protocol for experiment 3.4. A similar procedure to that of experiment 3.3 was adopted but the intracranial injections of 2-Dgal and saline were delayed until 20 min after training with the green LED.

Table 3.6: Summary of the data from experiment 3.4.

i.c. Treatment	i.p. Treatment	n	% avoiding LED	% avoiding chrome bead
2-Dgal	Lithium	27	59.3	14.8
saline	Lithium	25	76.0	28.0
2-Dgal	Saline	28	25.0	7.1
saline	Saline	25	32.0	8.0

Table 3.7: Summary of statistical comparisons of % avoiding LED and chrome bead.

Comparison*	Green LED		Chrome bead	
	χ^2	p≤	χ^2	p≤
(2-Dgal - LiCl) with (sal - LiCl)	0.98	NS	0.56	NS
(2-Dgal - Saline) with (sal - LiCl)	11.78	0.001	2.73	NS
(sal - Saline) with (sal - LiCl)	8.05	0.01	2.17	NS
(2-Dgal - LiCl) with (2-Dgal - Saline)	5.29	0.05	0.23	NS
(2-Dgal - LiCl) with (sal - Saline)	2.86	NS	0.11	NS

*(i.c. injection - i.p. injection)

Figure 3.6; The effect of delaying the injection of 2-Deoxy galactose, on sickness-conditioning.

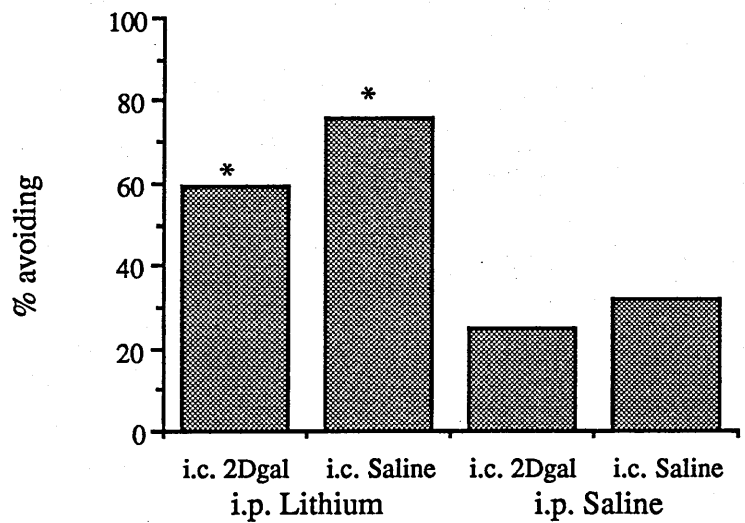


Figure 3.6; The effect of a delayed intracranial injection of 2-Dgal, 10 min before the induction of sickness by an i.p. injection of lithium. Both i.p. lithium injected groups contained significantly more chicks that avoided the green LED compared to the groups injected i.p. with saline ($p \leq 0.05$ and $p \leq 0.01$ respectively), indicating that learning took place in both groups injected i.p. with lithium. Thus the delayed i.c. injection of 2-Dgal did not induce amnesia for the task.

DISCUSSION

A conditioned aversion was produced in young chicks by an intraperitoneal injection of LiCl 30 min after pecking at a green LED or a chrome bead (Figure 3.1). The retention test, 4 hr later, consisted of a 10 sec presentation of the paired and unpaired stimuli. If the learning task were specific to one stimulus then birds that remembered the association would avoid the paired stimulus while all treatment groups would continue pecking at the unpaired stimulus. The results of the first experiment indicate that more than 70% of the birds are capable of learning a sickness-induced aversion to the green LED. Indeed, when the LiCl-injected birds were given the retention test it was noted that, as well as avoiding the bead, a number of the chicks displayed vigorous head shaking, a characteristic disgust response that is often evident when chicks are presented with noxious stimuli (Lee-Teng and Sherman, 1966; Cherkin, 1969). Only 10-20% of birds avoided the unpaired stimulus in the test, indicating that the majority of birds had made a specific association between pecking at the training stimulus and subsequent sickness. It should be noted that, by contrast with previous demonstrations of conditioned aversion in chicks (Gaston, 1977; Gaston, 1978) the stimulus paired with illness was a dry bead rather than a flavoured or coloured solution, thus ruling out the possibility that the association of the bead with subsequent sickness was the result of delayed, rehearsed or continued taste responses, as have previously been suggested to account for sickness-conditioning phenomena (Revusky, 1977).

The results imply that the chick initially forms a memory for the novel stimulus even without its specific pairing with reward or aversive consequences. It is half an hour after this initial memory that the characteristics of the green LED become associated with illness. It seems appropriate then, to suggest that the chick is capable of retaining a

short-term visual representation of the LED without strong reinforcement. The experiments reported here show that this memory must last for a period of at least thirty minutes, but not more than 60 min after training. After this period the memory is either lost or replaced by new information.

It is unlikely that the amnesia observed in experiment 3.3 was due to an interaction between the intracranial injection of 2-Dgal and the intraperitoneal injection of LiCl. Intracranial injection of LiCl caused amnesia for a passive avoidance task in chicks when injected before training (Benowitz and Sperry, 1973), however, in those experiments it had no effect when injected as little as 10 seconds after training. In rats, chronic (Hetmar and Nielson, 1988) but not acute (Maggi and Enna, 1980) administration of LiCl caused changes in binding of a number of different forebrain receptor ligands. Since in the present experiments, the acute dose of LiCl was administered intraperitoneally, half an hour after training, it is unlikely that the treatment had any confounding effect on brain processes. Moreover, the results from the experiment reported in figure 3.5 indicate that amnesia was no longer evident when 2-Dgal was administered 20 min after the presentation of the LED, a time that was still 10 min before the administration of LiCl.

The fact that 2-Dgal has been shown to disrupt memory for an event which did not have any obvious significance for the chick until half an hour later, when it became ill, indicates that a representation of the LED, despite providing no gustatory cues, must have been stored in the brain very soon after the chick pecked at it, by a process which required the synthesis of membrane glycoproteins. The processes taking place in sickness-conditioning do not follow traditionally recognised rules at the behavioural level. However the results suggest that the macromolecular processes necessary and sufficient for sickness-conditioning are similar to those for other forms of learning, since

2-Dgal has caused amnesia in both the passive avoidance task and the sickness-induced aversion task. Thus the common laws governing learning should be sought at the macromolecular level rather than the behavioural.

It could be that some kind of long term visual buffer was being employed to retain visual information about the characteristics of food previously eaten. The visual buffer would need to remain active long enough for the ingested food to become biologically significant (i.e. make the animal ill). The idea of a short term buffer in the chick brain is not new. The lesion studies by Horn and workers (Cippolla-Neto, Horn and Mc Cabe, 1982; Horn, 1981) suggest that the left IMHV is necessary for retention of the imprinting task but that the right IMHV is a short term buffer store that acts to consolidate a further long term store, S', somewhere else in the brain. While it is not suggested that memory of the CS in sickness-conditioning is held in the right IMHV and slowly fed to a permanent store elsewhere, the results reported here suggest that some temporary visual storage is available to the chick.

In the present experiment, a putative buffer could have been fooled into storing the visual characteristics of the LED without the chick having ingested food. If a visual buffer did exist the 2-Dgal could be acting to prevent the reading of information from buffer to long term memory, implying that a visual buffer requires the synthesis of fucosylglycoproteins. The drug may be doing this either by effectively erasing the buffer or by breaking the link between buffer and long term memory, thus preventing information from passing between the two. Following on from this hypothesis, it would make sense to perform lesion studies similar to those of Horn et al, before and after training chicks on the sickness-conditioned aversion paradigm described here. Initially bilateral lesions should be performed, followed by unilateral and possible serial unilateral lesions, as described by Horn (Horn, 1985).

Further to the experiments described here, unilateral injections of 2-Dgal will be performed to investigate lateral control of the sickness-induced aversion, in chapter 4. There also remains the possibility that 2-Dgal acted as an amnestic agent in the experiments described here, by becoming a cue upon which recall was dependent. This will be explored further in chapter 5.

CHAPTER 4. LATERALISATION OF THE FUNCTION OF FUCOSYLGLYCOPROTEINS IN LEARNING AND MEMORY IN THE DAY-OLD CHICK.

INTRODUCTION

Hemispheric lateralisation is widely accepted as a phenomenon common to many animals including humans, birds and small mammals (Andrew, 1983; Nottebohm, 1977; Denenberg and Yutzey, 1985). A recognised pattern has emerged from the clinical data indicating that the left hemisphere is concerned with the control of language and integral processing while the right hemisphere is concerned with holistic processing and emotion (Springer and Deutsch, 1981). Evidence also suggests that there are some sex differences in human laterality, particularly in the young. Boys between the ages of 6 and 13 tend to have a right hemisphere specialisation for spatial recognition tasks, while girls of a similar age have verbal specialisation (Witelson, 1976). Furthermore, emotional and fear responses tend to be initiated more by the right hemisphere in humans (Dimond, Farrington and Johnson, 1976), rats (Denenberg, 1981) and chicks (Andrew and Brennan, 1983; Phillips and Youngren, 1986).

Some of the most surprising lateralised results have been found in song control in canaries. If the left hypoglossus was cut before spring, the right hemisphere assumed dominance for song control. Unilateral lesions indicated that the caudal hyperstriatum ventrale (HVC) was the locus of control of song behaviour. By tracing the connections to this area, area X of the parolfactory lobe and a round nucleus in the archistriatum (RA) were also implicated in song control. The most important aspect of these results was that

lesions of the left hemisphere caused a greater detriment to song than lesions of the right hemisphere (Nottebohm, 1977). Strangely, the asymmetries in the volume of HVC and RA were not evident in more recent morphological studies (Nottebohm, Nottebohm and Crane, 1986). Golgi studies characterised the hormone sensitive type IV neurons of the RA in female canaries, indicated that during hormone induced song development, new synapses were added throughout the dendritic tree. Unfortunately, interhemispheric comparisons were not made in this study (Canady et al, 1988).

The most parsimonious explanation for lateralised function is that of economy of processing and storage space within the brain. This explanation does not, however, deal with the problem of why there should be two hemispheres at all. Furthermore it does not explain why trends in lateralisation appear to be common across species. Why, for instance, is speech in humans and song control in canaries, lateralised to the left hemisphere? It seems unlikely that this is pure coincidence and is more likely to be evidence of lateralised function in a common ancestor. If the lateralisation of certain brain functions was determined so early in evolutionary history this suggests that there is some fundamental role for the pattern of brain organisation common across many species.

In attempting to determine the function of lateralisation, it is necessary to investigate which functions are lateralised and which are not. In this respect the young chick is an excellent model for the investigation of laterality. The visual system is virtually completely decussated (Cowan, Adamson and Powell, 1961) such that the input from one eye is directed exclusively to the contralateral hemisphere. This enables the experimenter to block the direct visual input to one hemisphere simply by occluding the contralateral eye with a patch, leaving the visual input to the ipsilateral hemisphere unchanged. Thus the performance of individual hemispheres in any visual task can be

compared. Using this technique Richard Andrew (Andrew et al, 1980) demonstrated lateralisation of learned visual discrimination in young chicks. The task was learned faster when the left eye was occluded, indicating left hemisphere dominance. Andrew also demonstrated that chicks display a heightened fear response to novel stimuli following occlusion of the right eye, indicating a right hemisphere locus for the control of this behaviour. Feeding and sexual behaviour are also lateralised, this is determined during development by light input to the right eye, some time before the chick hatches (Rogers, 1982). Sex differences in lateralisation of fear have also been discovered (Andrew and Brennan, 1984), and a sex difference in lateralisation of position learning has recently been found (Vallortigara, Zanforlin and Cailotto, 1988). Development of sex differences in laterality are controlled by the levels of testosterone in the young chick (Zappia and Rogers, 1987), but it is not understood why such profound sex differences in laterality should be found, or why there are such sharply timed changes in hemispheric dominance during the early development of the young chick (Andrew and Brennan, 1985).

In combination with the eye patch technique, unilateral administration of drugs can also be performed. The unossified skull of the day-old chick enables intracranial administration of a drug directly into brain tissue. A Hamilton syringe can be used, with a long needle fitted with a plastic sleeve that acts as a stopper, holding the depth of injection constant. When a tritiated form of the drug was injected in this way it stayed within the confines of the designated hemisphere (Howard, Rogers and Boura, 1980). By injecting some metabolic inhibitor such as a protein synthesis inhibitor, behavioural comparison of the performance of left and right hemisphere-injected birds may bring to light the different roles of the two hemispheres (Rogers and Anson, 1979). In a series of behavioural tests, unilateral injections of glutamate or cycloheximide were used to demonstrate lateral control of visual discrimination learning, auditory habituation, attack and copulatory

behaviour.

A number of lateralised phenomena have also been discovered in young chicks, using an imprinting task. Initial evidence of asymmetry related to imprinting came from an electron microscope study of the IMHV (Bradley, Horn and Bateson, 1981). Training was associated with a number of changes in the synaptic structure in the left IMHV but not the right. With partial training, synaptic apposition zones were longer in the right hemisphere compared to the left but this difference disappeared after further training. Unfortunately, previous biochemical studies in the imprinting task did not make a distinction between samples from left and right hemisphere (Bateson, Rose and Horn, 1973; Horn, Rose and Bateson, 1973).

Once asymmetries in the chick brain had been identified after imprinting, lesion studies were performed. When unilateral lesions were made to the IMHV before training the chicks acquired a preference equally well whether lesioned in the left or the right IMHV (McCabe, Horn and Bateson, 1981). A serial lesion study was then carried out (Cipollaneto, Horn and McCabe, 1982). Chicks lesioned in the right IMHV, trained and then lesioned in the left IMHV, lost their acquired preference for the training stimulus, while those lesioned in the left IMHV before training retained the acquired preference after a lesion to the right IMHV. The results implied that the left IMHV had a permanent storage function while the right IMHV was impermanent. However, when the left IMHV was removed after training the chicks were not amnesic, indicating that the left IMHV was not needed for recall.

Horn formulated a model based on these findings, in which the left IMHV acts as a permanent store while the right is a buffer store that slowly feeds information to some

other permanent store, S' (Horn, 1985). However, when the post training lesion, to the right IMHV, was delayed until 20 hours after training, the chicks did not retain a preference (Horn, McCabe and Cipolla-Neto, 1983). Horn concluded that S' could not function independently of the left IMHV.

Results from recent lesion studies with the passive avoidance task have suggested that the IMHV is required for acquisition and for a short time after training but is no longer needed once memory has consolidated (Patterson, Gilbert and Rose, 1990), this work also suggests that the right and left IMHV have different roles in passive avoidance learning.

Biochemical studies following passive avoidance training in rats and chicks (Rose and Jork, 1987, Mc Cabe and Rose, 1985) have implicated fucosylglycoproteins in memory storage for this task. Fucose is almost exclusively incorporated into the end chain of glycoproteins destined for addition into the synaptic plasma membrane (Burgoyne and Rose, 1980). Fucose incorporation was increased following passive avoidance training and the increase was localised to the right forebrain base (Mc Cabe, 1985). However, a lateralised role of fucosylated glycoproteins has not yet been confirmed behaviourally. The following experiments utilise the technique of unilateral hemispheric administration of 2-Deoxy galactose in order to determine whether the function of glycoprotein synthesis, in the two tasks described in chapters 2 and 3, is lateralised.

Experiment 4.1; Testing the effect of unilateral administration of 2-Deoxy galactose on serial colour-discrimination passive avoidance learning.

METHOD

A total of 484 day-old chicks of both sexes was used in a balanced design with 27 separate replications. Each day, chicks were taken from a communal incubator and placed in pairs in metal pens as described earlier. Before the start of the experiment the chicks were left undisturbed for 1.5 hr to allow them to acclimatise to the training pens.

At the start of the experiment each chick was given a 10 μ l intracranial injection of either 0.9% saline (Sal) or 10 μ mol 2-Deoxy galactose (2-Dgal) into either the left or the right hemisphere. The vials containing 2-Dgal and Sal were identical and coded without the knowledge of the experimenter, thus injection was performed "blind." Injections were administered freehand using a Hamilton syringe fitted with a stopper to control the depth of injection to 4 mm below the skull. A pilot study using tritiated 2-Dgal indicated that the drug does not pass into the contralateral hemisphere for at least 3 hr after injection (see appendix 3).

Directly after injection the chicks were pretrained in the usual way. On each occasion the red LED was presented for 20 sec followed quickly by a 20 sec presentation of the green LED, as described in chapter 2. Each pretraining trial was separated by a 15 min interval.

Training took place 45 min after injection and involved a single 20 sec presentation of the green LED dipped in methylantranilate, to each chick in turn. Those birds that did not peck at the training LED or did not peck at least once at each colour during pretraining,

Figure 4.1; Schematic representation of the protocol of experiment 4.1.

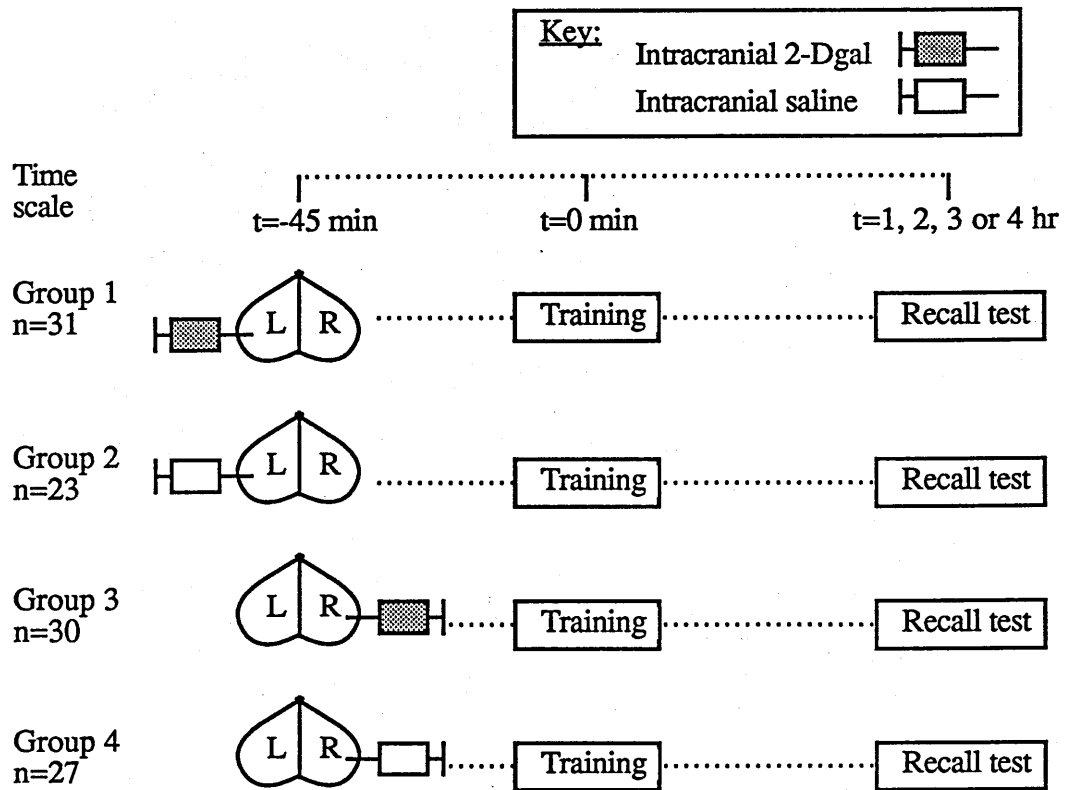


Figure 4.1; A schematic representation of the protocol of experiment 4.1. Four groups of chicks were injected unilaterally with either 2-Dgal or saline, into either the right or left forebrain hemisphere, 45 min before training. Each chick was given a retention test, 1, 2, 3 or 4 hr after training. No chick was tested more than once. The results are illustrated in figure 4.2.

were rejected at the end of the experiment. About 30% of individuals were rejected in this way and are not represented as part of the total N.

Each chick was given a retention test at either 1, 2, 3, or 4 hr after training. No chick was tested more than once. The retention test consisted of a 20 sec presentation of the red LED followed quickly by a 20 sec presentation of the green LED. During the retention test the "peck latency" of each bird to each LED was measured using a stopwatch, as described earlier. Those chicks that avoided the LED were assigned a peck latency of 20 sec. Since lateralisation phenomena in the young chick may vary between sexes (Andrew and Brennan, 1984) the sex of each chick was determined at the end of the experiment by using the wing feather method (Hann, 1966). This method determines the sex of each chick by a developmental difference between the male and female flight feathers; the male feathers develop slightly later than the female, which tend to have two rows, while the males only have one. The procedure was repeated over a number of days in a balanced design until there were approximately 35 individuals (34 ± 7) in each of the four groups (actual n's are given in figure 4.1).

The results were analysed by survival analysis as described previously. Overall comparisons were made between sex as well as individual comparisons between each of the four treatment groups for each retention time.

RESULTS

The treatment groups will be abbreviated to "2-Dgal - LH" for 2-Deoxy galactose injected into the left hemisphere, and "Sal - RH" for saline injected into the right hemisphere.

Each drug treatment group was compared with its respective saline control. Thus the performance of chicks injected with 2-Dgal into the left hemisphere was compared with that of chicks injected with saline into the left hemisphere. Also, comparisons were made between hemispheres for similar treatments. A summary of the Survival Analysis comparisons is given in tables 4.1 and 4.2.

Table 4.1; Summary of p values from multiple survival analyses (data for green LED).

Treatment comparison	Recall test (time after training)			
	1hr	2hr	3hr	4hr
(2-Dgal - LH) with (Sal - LH)	-	-	-	p=0.028
(2-Dgal - RH) with (Sal - RH)	p=0.002	p=0.003	p=0.073	p=0.015
(Sal - LH) with (Sal - RH)	-	p=0.036	-	p=0.074
(2-Dgal - LH) with (2-Dgal - RH)	-	-	-	p=0.098

"-" = no significant difference; "2-Dgal"=i.c. 2-Deoxy galactose; "Sal"=i.c. saline

"LH"=left hemisphere injection; "RH"=right hemisphere injection

Figure 4.2a; Comparison of green LED data from chicks injected into the left hemisphere.

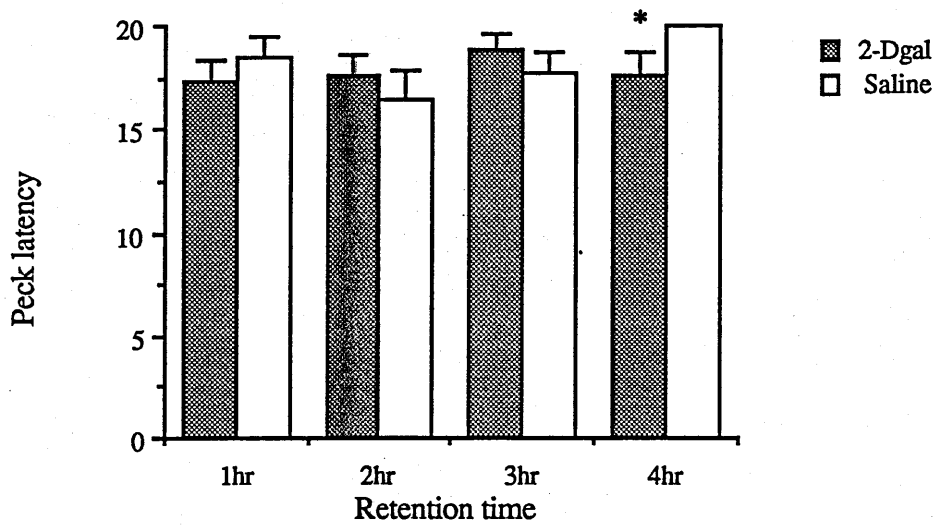


Figure 4.2b; Comparison of green LED data from chicks injected into the right hemisphere.

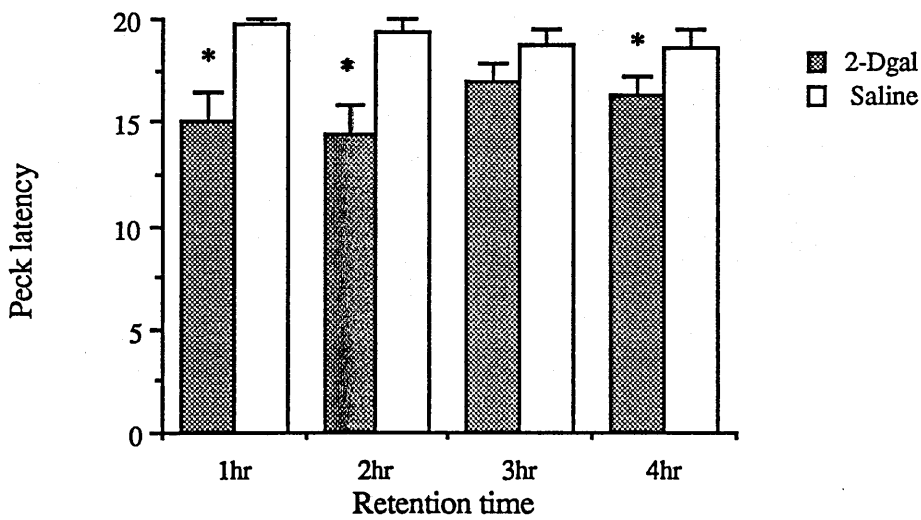


Figure 4.2; A comparison of the green LED data from chicks injected with saline or 2-Dgal into the left or right hemisphere. Figure 4.2a represents data left hemisphere chicks while 4.2b illustrates data from the right hemisphere birds. The error bars represent the standard error mean of each sample. The chicks injected with 2-Dgal into the left hemisphere had significantly lower peck latencies than those injected with saline, 4 hr after training ($p=0.028$). Significant differences were found between the mean peck latencies of those groups injected saline and 2-Dgal into the right hemisphere, 1 hr, 2 hr and 4 hr after training ($p=0.002$, $p=0.003$ and $p=0.015$ respectively) but not 3 hr after training ($p=0.073$).

Table 4.2; Summary of p values for multiple survival analyses (data for red LED).

Treatment comparison	Recall test (time after training)			
	1hr	2hr	3hr	4hr
(2-Dgal - LH) with (Sal - LH)	-	-	-	-
(2-Dgal - RH) with (Sal - RH)	p=0.031	-	p=0.009	-
(Sal - LH) with (Sal - RH)	-	-	-	-
(2-Dgal - LH) with (2-Dgal - RH)	-	-	p=0.004	p=0.045

"-" = no significant difference; "2-Dgal"=i.c. 2-Deoxy galactose; "Sal"=i.c. saline

"LH"=left hemisphere injection; "RH"=right hemisphere injection

The 2-Dgal - RH group had a significantly shorter peck latency to the green LED compared to the Sal - RH group 1 hr, 2 hr and 4 hr after training ($D=9.437$, $p=0.002$; $D=8.977$, $p=0.003$; $D=5.914$, $p=0.015$ respectively). There was also a significant difference in peck latency to the green LED between the 2-Dgal - LH and Sal - LH groups, 4 hr after training ($D=4.809$, $p=0.028$), this difference may have been due to the zero variance of observations in the saline group at that time point. However, the peck latencies of groups injected with 2-Dgal and saline into the right hemisphere were not significantly different, 3 hr after training ($p=0.073$).

Figure 4.3a; Comparison of red LED data from chicks injected into the left hemisphere.

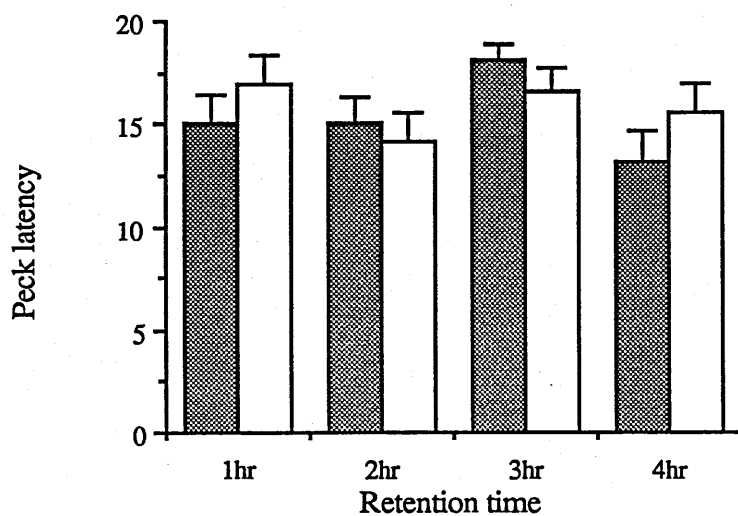


Figure 4.3b; Comparison of red LED data from chicks injected into the right hemisphere.

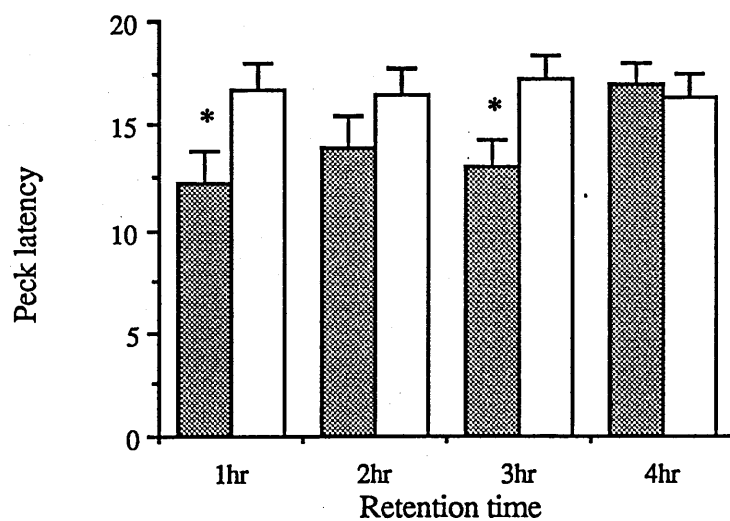


Figure 4.3; A comparison of red LED data from those chicks injected with saline and 2-Dgal into the left or right hemisphere. Figure 4.3a represents data from left hemisphere chicks while 4.3b illustrates data from the right hemisphere chicks. The error bars represent the standard error mean of each sample. There were significantly shorter peck latencies to the red LED, 1 and 3 hr after training, for those chicks injected with 2-Dgal into the right hemisphere ($p=0.031$ and $p=0.009$ respectively). No other differences were significant.

There were differences in the peck latency to the red LED between the 2-Dgal - RH and Sal - RH groups, 1 hr and 3 hr after training ($D=4.63$, $p=0.031$; $D=6.78$, $p=0.009$ respectively). No significant differences between the two sexes could be found in any of the treatment groups.

The above method of analysis has the disadvantage that multiple pair-wise comparisons increase the probability of making at least one type I error. There is however, little alternative if peck latency is to be used as a measure. If the data are expressed as % avoiding then a 3-dimensional χ^2 analysis may be performed between the factors of Treatment, Time and Number Avoiding. The data for the green LED presentations have been reproduced in table 4.3.

Table 4.3: % avoiding data from the green LED presentations of experiment 4.1.

Treatment Group	Recall test (time after training)			
	1hr	2hr	3hr	4hr
Left - 2-Dgal	76.6	79.3	86.7	76.7
Left - Saline	91.3	77.8	80.0	100.0
Right - 2-Dgal	63.3	64.5	73.2	61.8
Right - Saline	96.3	96.6	90.6	90.3

The 3-dimensional χ^2 analysis revealed that Time was independent of both Treatment and

Number Avoiding ($\chi^2=15.75$, with 21 degrees of freedom). Thus, the % avoiding score did not vary significantly with time in any treatment group. Further 2-way χ^2 analyses were carried out by collating the data with respect to recall time. Comparisons between treatment groups indicated that the scores of the left hemisphere 2-Dgal group and the left - saline group were not significantly different ($\chi^2=0.98$), while a comparison between the groups injected into the right hemisphere indicated a significant difference ($\chi^2=26.33$, $p\leq 0.001$). Thus, 2-Dgal injected into the right hemisphere before training gave rise to amnesia for the task, while injection into the left did not.

DISCUSSION

The results suggest that when glycoprotein synthesis was inhibited in the right hemisphere during training, the chicks were amnesic between 1 and 2 hr after training, and 4 hr after training. This amnesia was not evident 3 hr after training, although the results indicated a trend towards a significant difference ($p=0.073$). When glycoprotein synthesis was inhibited in the left hemisphere during training, no amnesia was evident until 4 hr later ($p=0.028$).

This time course of amnesia is curious. It implies that the right hemisphere requires the synthesis of fucosylglycoproteins more than the left, for memory formation. Since the time course of activity of 2-Dgal is not clearly understood, it is difficult to determine what is implied by the dip at the 3 hr time point in the right hemisphere-injected group. However, there does appear to be a general emphasis on the synthesis of fucosylglycoproteins in the right hemisphere, more than the left, at least in the earlier part of long term memory formation, between 1 and 3 hr after training.

The results agree with previous findings that fucose incorporation increased in the right forebrain base after training (McCabe and Rose, 1985). Since many other studies on passive avoidance training have shown left hemisphere lateralisation, it seems unlikely that fucosylated glycoproteins form the main molecular substrate of memory retention. It is more likely that 2-Dgal has disrupted one aspect of the consolidation process. Fucosylated glycoproteins may have some connection with the putative buffer store in the right IMHV suggested by the lesion studies of Horn et al (Horn, 1985). Horn suggests that the buffer slowly feeds a long term store S', somewhere else in the forebrain, 2-Dgal may have acted to disable this process in some way, perhaps by blocking communication between respective cells and thus preventing communication of information to the appropriate storage area. However, a more recent lesion study using the passive avoidance task has also implicated the left IMHV as a buffer, which is required for acquisition of the task but not long term retention (Patterson, Gilbert and Rose, 1990).

The effect described here may be due to 2-Dgal disabling a function of memory at a time when the right hemisphere is more important than the left. If this is the case then it indicates that the effect of 2-Dgal is chronologically more discrete than has previously been suggested and that unilateral administration of the drug at other times with respect to training may yield a left hemisphere effect rather than a right one. It is, however, difficult to predict what those times may be.

An alternative interpretation of the result is that fucosylated glycoproteins play a role in storing the emotional aspects of memory retention (if it is reasonable to hypothesise that different aspects of a memory are stored in different areas of the brain or by different molecular substrates; see Denenberg and Yutzey, 1985; Denenberg, 1981). The passive avoidance training experience has a large emotional component and it has been shown

that the fear response of young chicks is lateralised to the right hemisphere (Andrew and Brennan, 1983). The drug may be acting to disarm only this aspect of the experience, by preventing a fear response from being initiated by the right hemisphere. Although this hypothesis does not match well with the biochemical evidence that the fucose incorporation is greatest in the right forebrain base after passive avoidance training (McCabe and Rose, 1985).

There is little way of testing the latter hypothesis directly. However, if the drug is acting by some anxiolytic or psychotropic mechanism, this may be tested by measuring whether the learning is state dependent. Many psychotropic drugs have been shown to cause apparent amnesia indirectly, by altering the psychological state of the experimental animal during learning. This drug action can be revealed by readministration before a recall test. In the case of state dependent amnesia, readministration of the drug will normally allow recall. This possibility is explored in chapter 5.

Experiment 4.2; Testing the effect of unilateral administration of 2-Deoxy galactose on sickness-conditioned aversion learning.

INTRODUCTION

Little study has been made of lateralisation in sickness-conditioning, particularly using pharmacological techniques. Denenberg et al (1980) classified the task as a learned fear and hypothesised that control of the behaviour would be centered in the right hemisphere because other affective behaviour in rats has been localised to that hemisphere (for a review, see Denenberg, 1981). Since fear responses are lateralised to the right hemisphere in chicks (Andrew and Brennan, 1983) it follows that unilateral inhibition

should yield similar results.

There is still controversy over what is the central control of this type of learning. In a review of taste aversion learning, Gaston (1978a) claims that cortical participation is generally not required for this type of learning in rats. Unilateral and bilateral KCl-induced cortical spreading depression (Best and Zuckerman, 1971) did not affect sickness-conditioning in rats. In contrast, Leher and Nachman (1973) also applied topical KCl unilaterally to cause cortical spreading depression during a one trial conditioned taste aversion. The learned response was not evident in the untrained hemisphere, providing evidence that a one trial test may be cortically mediated. However, spreading depression alone may condition a taste aversion to saccharin (Freedman and Ward, 1976), thus the technique has probably yielded ambiguous results.

The results reported in Chapter 3 suggest that 2-Dgal abolished sickness-conditioning in chicks. It is unlikely that 2-Dgal affects any other area apart from the forebrain when injected by the method described earlier, indicating a telencephalic control mechanism. Other drug studies also indicate that there is some central control mechanism for delayed aversion learning. Cycloheximide induced amnesia for a taste aversion in rats (Tucker and Gibbs, 1979), indicating that macromolecular synthesis is necessary for memory retention in this task.

The following experiment attempts to discover whether unilateral administration of 2-Dgal to the chick forebrain can induce amnesia for the sickness-conditioned aversion paradigm, in the same way as for the passive avoidance paradigm.

METHOD

A training method similar to that described in Chapter 3 was adopted. A total of 254 day-old chicks were taken over a period of 11 days and placed in the training pens in pairs. No food was made available to them during the course of the experiment. After the usual acclimatisation period each chick was pretrained with a small white bead and trained 20 min later by allowing it to peck at the green LED for 10 sec. 10 minutes before training each chick was given a unilateral intracranial injection of either 2-Dgal or saline. A schematic representation of the experimental protocol is given in figure 4.4. Those birds that did not peck during the 10 sec training period were rejected at the end of the experiment.

On each day of the experiment the chicks were divided into eight treatment groups. Four groups were injected i.p. with lithium chloride (0.1ml of 1mol) and the other four groups were injected i.p. with a similar volume of normal saline. The i.p. injections were administered 30 min after training. The chicks in each treatment group were injected intracranially with 10 μ l of either 2-Dgal (10 μ mol) or 0.9% saline into either the left or right hemisphere, as in experiment 1. The i.c. injections were administered 10 min before training.

The chicks were given a retention test as described in Chapter 3, 4 hr after training. The behaviour of chicks was recorded as "peck" or "avoid" and the results were expressed as % avoiding. As usual both the drug and saline vials were "blind" coded to cancel any bias that may have been induced by the experimenter.

Figure 4.4; Schematic representation of the protocol of experiment 4.2.

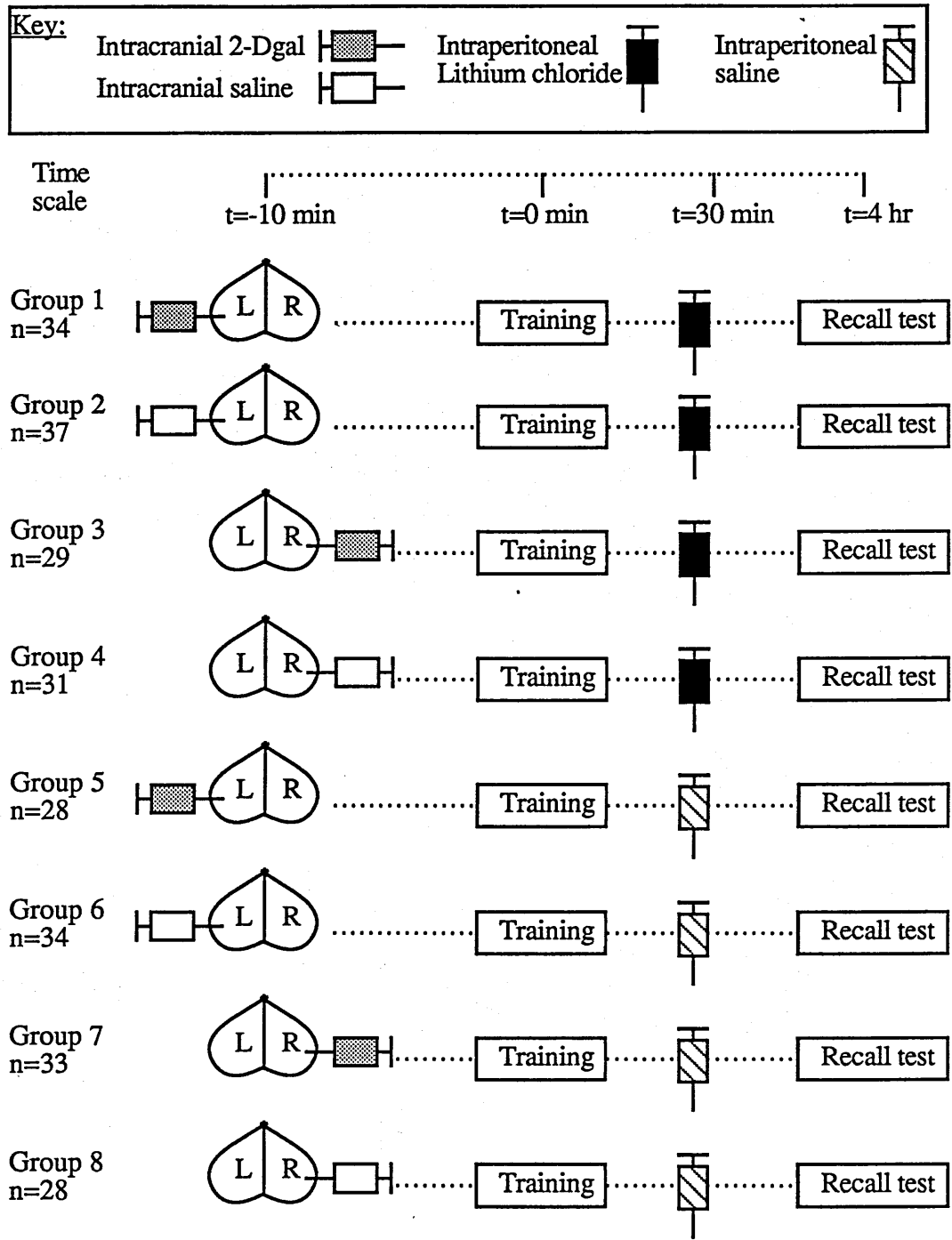


Figure 4.4; Eight groups of chicks were injected i.c. with either 2-Dgal or saline into either the left or right forebrain. The chicks then trained and injected i.p. with either LiCl or saline. Results are illustrated in figure 4.5.

RESULTS

A summary of the data is given in table 4.3 and the results are shown graphically in figure 4.5. Summaries of χ^2 comparisons are given in tables 4.4 and 4.5. χ^2 tests were performed to compare individual groups. No differences in the % avoiding the chrome bead (unpaired stimulus) were found between any of the eight groups; chrome bead scores ranged from a minimum of 7% to a maximum of 27% avoiding.

Table 4.3 ; A summary of the data from experiment 4.2.

Treatment		n	% avoiding	
			green LED	chrome bead
i.p. LiCl:	Left hemisphere-2-Dgal	34	47	21
	Left hemisphere-Saline	37	51	27
	Right hemisphere-2-Dgal	29	52	17
	Right hemisphere-Saline	31	61	16
i.p. Saline:	Left hemisphere-2-Dgal	28	18	11
	Left Hemisphere-Saline	34	35	12
	Right Hemisphere-2-Dgal	33	30	18
	Right Hemisphere-Saline	28	25	7

There are two possible sets of statistical comparisons that can be made with this data. The first is to compare i.p. lithium and i.p. saline groups with similar i.c. injections into

similar hemispheres (see table 4.4). Alternatively, the groups injected i.c. with 2-Dgal can be compared with those injected i.c. with saline into similar hemispheres, with similar i.p. injections (see table 4.5)

Table 4.4: Statistical comparison of i.p. lithium and i.p. saline injected groups with similar i.c. treatments.

i.c. injection site	i.c. treatment	χ^2	p≤
Left hemisphere	2-Dgal	4.62	0.05
Left hemisphere	Saline	1.26	NS
Right hemisphere	2-Dgal	2.09	NS
Right hemisphere	Saline	6.46	0.05

There was a difference between the i.p. lithium and i.p.saline treated groups that were injected i.c. with saline into the right hemisphere ($p \leq 0.05$), indicating that learning had taken place in the lithium injected group. A difference between lithium and saline injected birds was not found after they had been injected with 2-Dgal into the right hemisphere ($\chi^2=2.09$). This result could be interpreted as amnesia in the 2-Dgal injected group, however, when the left hemisphere-injected groups were compared, there was a difference between the lithium and saline i.p. groups when treated with 2-Dgal ($p \leq 0.05$) but not when treated with i.c. saline ($\chi^2=1.26$). This brings the validity of the comparison under question since it implies that only 2-Dgal caused amnesia when injected into the right hemisphere but only saline caused amnesia when injected into the left hemisphere.

Figure 4.5; Effect of unilateral administration of 2-Deoxy galactose on sickness-conditioned aversion learning.

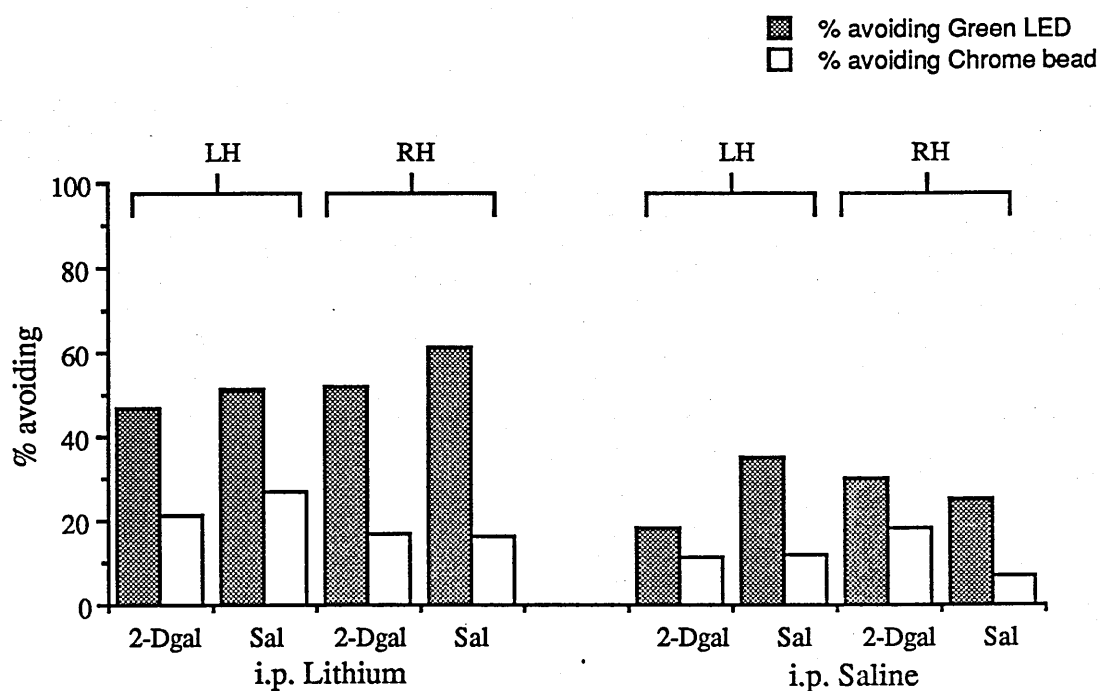


Figure 4.5; The effect of unilateral injection of 2-Dgal on sickness-conditioning. Eight groups of chicks were injected unilaterally i.c. with either 2-Dgal or saline (LH=left hemisphere injection, RH=right hemisphere injection). 30 min after training, half the chicks were injected i.p. with either LiCl or saline. No significant differences were found between i.c. 2-Dgal and saline injected groups, with similar i.p. treatments, indicating that the 2-Dgal did not cause amnesia for the task, whether injected into the right or left hemisphere.

Table 4.5: Statistical comparison of i.c. saline with i.c. 2-Dgal injected groups, that had similar i.p. treatments.

i.p. treatment	Site of i.c. injections	χ^2	p
Lithium chloride	Left hemisphere	0.02	NS
Lithium chloride	Right hemisphere	0.24	NS
Saline	Left hemisphere	1.56	NS
Saline	Right hemisphere	0.03	NS

When a comparison of scores from groups given similar i.p injections and injected i.c. into similar hemispheres (table 4.4) no differences were found between i.c. saline and 2-Dgal groups. These results further indicate that the 2-Dgal has had no appreciable effect, whether injected into the left or right hemisphere. Furthermore, an overall χ^2 comparison of those groups injected i.p. with lithium indicates no significant difference between groups ($\chi^2=1.4$).

DISCUSSION

In experiment 4.2 an attempt was made to discover whether amnesia induced by 2-Dgal was lateralised in the sickness-conditioned aversion paradigm in the same way that it was in the passive avoidance discrimination paradigm. Although the results shown in table 4.4 indicate that 2-Dgal injected into the right hemisphere caused amnesia, there is some doubt that this is a valid result, since further comparisons indicate that saline injected into

the left hemisphere also caused amnesia while 2-Dgal did not.

The results of a more valid comparison are given in table 4.5. The results demonstrate that no i.c. injection of the 2-Dgal caused amnesia. Referring to chapter 3, 2-Dgal caused substantial amnesia in chicks performing this task when injected bilaterally. It may be that a certain total dose of 2-Dgal is required to inhibit learning for this task. It would be desirable to repeat the experiment using a total dose of 2-Dgal, equivalent to that used in the bilateral injection experiment (20 μ mol) of chapter 2. This amount of 2-Dgal injected into one hemisphere only may, however cause undesirable behavioural side effects.

The present result indicates that only one hemisphere is required to learn the task, but it appears that either hemisphere is capable of learning the task. Although the result is disappointing it agrees with previous work that has attempted to localise a center for sickness-conditioning. In many mammalian studies there has been no consistent cortical center identified as a locus of conditioned aversion learning, although the lateral and ventromedial hypothalamus and amygdala have been consistently implicated (Gaston, 1978a).

It may be possible that there are a number of areas responsible for encoding the memory and that a loss of one hemisphere does not interfere with recall, or alternatively, that if one area of the brain is prevented from learning the task, a second, unaffected area, will adopt the function of learning the aversion.

A study using the eye-patch technique, in which one of the chick was occluded, yielded similar results. Monocularly trained chicks acquired illness-induced food aversions to novel coloured and flavoured food, however, if they were tested with the untrained eye

the aversion was mediated by the novel flavour only (Gaston, 1980). The possibility that either hemisphere is capable of learning this task has been demonstrated by other workers. The flexibility of the chick brain in sickness-conditioning was further demonstrated by interhemispheric transfer of the learning, in which recall was evident in the untrained hemisphere (Gaston, 1978b). In rats, the right hemisphere was dominant for the acquisition of a taste aversion, but only if the rats were handled during infancy (Denenberg and Yutzey, 1985).

A further investigation, using the eye patch technique could be employed to confirm the results reported here. Also, by using a post-training lesion technique, it would be possible to determine what hemisphere is more important to recall. Pretraining lesions should also be performed to determine which, if any, areas of the forebrain are required for memory retention of sickness-conditioning.

CHAPTER 5. TESTING 2-DEOXY GALACTOSE FOR A STATE DEPENDENT ACTION.

INTRODUCTION

The ability of an animal to retrieve the memory of a specific experience is curiously dependent upon the operational "state" of its central nervous system (CNS) at the times of acquisition and retrieval. If the operational state of the animal's CNS at the time of a retrieval test is markedly different from that at the time of acquisition, an apparent amnesia may be observed. This mismatch between the animal's general state of arousal, or perception, at acquisition and retrieval test has been called state dependence or dissociation. State dependent recall can be brought about by the application of some drugs during either the acquisition or retrieval period (Overton, 1974), or by some other state altering treatment such as, temperature extreme (Richardson et al, 1984; Hinderliter, Webster and Riccio, 1975) or time of day (Holloway, 1978; Overton, 1978; Riccio and Richardson, 1984). The effect has been demonstrated in many species including humans (Weingartner, 1978).

The state dependence phenomena was first noticed by Girden and Culler (1937) when they conditioned autonomic and skeletal motor responses in animals injected with curare. When the animals were subsequently tested in a non-drugged state no evidence of conditioning was found. However, when the drug was readministered the conditioned responses were again present. Since then many other drugs have been found to cause dissociated learning giving rise to state dependent retention (Overton, 1974), including alcohol (Deutsch and Roll, 1973), combinations of alcohol, caffeine and nicotine (Lowe,

1988); chlorpromazine (Grossman and Miller, 1961), scopolamine (Berger and Stein, 1969), morphine (Crowder et al, 1972), marijuana (Kubena and Barry III, 1972) and pentobarbital (Overton, 1964).

From the impressive list of drugs that have been found to cause state dependent recall, one might assume that many if not all centrally acting drugs have a state dependent quality. However, other drugs do not cause amnesia by state dependence, for example, glutamate and ouabain (Lee et al, 1989). Recently there has been some controversy over the action of the protein synthesis inhibitor anisomycin, which was reported to induce state dependent amnesia in chicks for a passive avoidance task (Bradley and Galal, 1987). A joint investigation showed that the amnesia caused by anisomycin was only state dependent when injected intraperitoneally and was not state dependent when the drug was administered via the more traditional intracranial route (Patterson, Rose and Bradley, 1989). Also, intraperitoneal administration of anisomycin did not cause a significant inhibition of brain protein synthesis and therefore the state dependent amnesia was due to some peripheral action of the drug. The state dependent action of a drug may be different according the method of administration. Discrepancies between state dependence investigations may also have further explanations in the drug dose needed to give a state dependent result (Eich, 1980). Often, state dependence investigations use a dose of drug that is large enough to cause behavioural effects other than apparent amnesia, such as drowsiness or gastrointestinal trauma, which may have confounding effects on the performance of animals during training and testing.

Many theories have been put forward to explain state dependent phenomena (Overton, 1978). A popular behavioural explanation is that the presence of a drug becomes a secondary conditioning stimulus for the learned response. The behavioural response

indicating recall will only be expressed when all contextual cues, including the drug, are reintroduced to the animal. In the case of drugs which are only state dependent at very high doses, causing unpleasant side effects to the experimental animal, it could be that the side effects of the drug become contextual cues for learning. If this is the case then recall will only take place when the same contextual cues (i.e. the same side effects) are again experienced by the animal.

The common method used to test an amnestic agent for a state dependent action is by using the 2×2 design shown in table 5.1 (Overton, 1974). The drug and control treatments are administered twice, once prior to training and once prior to testing. It is important to leave a similar time delay between drug administration and recall test, as was left between drug administration and the training trial, particularly if the drug acts by competing with a metabolic precursor, such that its active effect takes place some time after its administration.

Table 5.1; Standard protocol for a state dependence experiment, with the predicted outcome for a state dependent drug.

Acquisition treatment	Recall treatment	Predicted outcome
Drug	Control	Amnesia
Drug	Drug	Recall
Control	Control	Recall
Control	Drug	Amnesia or recall *

* See text

The drug has been shown to have a state dependent action if recall is observed in the Drug-Drug group when compared with the other groups. The Control-Drug group is also of importance since it will indicate if administration of the drug causes a retrieval deficit, in which the drug had no effect on learning but blocked recall for the task. The predicted outcome for this group may vary. If recall is observed in the Control-Drug group, this is referred to as asymmetrical dissociation (Overton, 1974). This result has been found for alcohol (Deutsch and Roll, 1973), physotigmine and scopolamine (Gardner et al, 1972) and some benzodiazepines (McNaughton, 1985) and may indicate that the drug interferes with acquisition but not recall. Alternatively the Control-Drug group may display amnesia, this is referred to as symmetrical dissociation or true state dependence.

Although state dependence, particularly that produced by drugs, is an interesting and little understood phenomenon, it is viewed here as a confounding complication in the investigation. The amnestic agent 2-Deoxy galactose, has been given prior to training because it is known to have a specific biological action in the brain. Since the drug has been shown to cause amnesia it is inferred that the molecular process it interfered with during training or after, is specifically involved in the formation of memory. However, there remains a possibility that 2-Dgal produced amnesia by a state dependent action; thus the memory was intact but could not be retrieved after the drug was metabolised. This would imply that 2-Dgal did not prevent memory retention by interfering with a molecular process directly involved in memory formation and is therefore not a true amnestic agent.

2-Dgal was tested for a state dependent action in the serial discrimination passive avoidance paradigm and in the sickness-conditioned aversion paradigm. The work using the sickness-conditioned aversion paradigm, reported in experiment 5.2, was a control

experiment that made up part of a series of collaborative experiments with Dr David Gilbert, reported in chapter 2 and published as part of a paper (Barber, Gilbert and Rose, 1989).

Experiment 5.1. Testing 2-Deoxy galactose for a state dependent action in the serial colour-discrimination passive avoidance task.

METHOD

175 day-old chicks of both sexes were split into five treatment groups in 10 balanced replications. The first four treatment groups followed the 2×2 protocol described earlier, while the fifth group was injected with saline both before training and before testing. Training in the fifth group consisted of a presentation of a green LED dipped in water instead of methylantranilate. The group was included to ensure that the continued intracranial injections did not lead to peck suppression, thus invalidating the results.

The chicks in groups 1 to 4 were pretrained and trained in the usual way, by a 20 sec presentation of the green LED coated in methylantranilate, having first been administered with bilateral intracranial injections of either 0.9% saline or 2-Dgal (10 µmol dissolved in 10 µl 0.9% saline in each hemispheric injection). Because the injections were performed prior to pretraining, the birds were trained approximately 45 min after injection.

After training the chicks were left undisturbed for 4 hr and 15 min. Then each chick was given a second injection of either 0.9% saline or 2-Dgal. The retention test followed 45

min after the second injection, since that was the time delay between the pretraining injection and training. The retention test involved a 20 sec presentation of the red LED followed by a 20 sec presentation of the green LED and the "peck latency" of each chick was recorded for each LED. Administration of injections was performed "blind" by having a second person code the two identical vials containing the 2-Dgal and saline.

The protocol used for the five treatment groups is illustrated in table 5.2 and displayed schematically, along with the n for each experimental group, in figure 5.1. The results were expressed as mean peck latency and are illustrated in figure 5.2. The data was analysed by the SPSSx Survival Analysis package held on the Walton Hall VAXcluster mainframe computer.

Table 5.2: Protocol for experiment 5.1, with predicted outcomes, assuming state dependence.

Group	Treatment			Prediction
	Pretraining	Training	Preretention test	
1	2-Dgal	MeA	Saline	Amnesia (pecking)
2	2-Dgal	MeA	2-Dgal	Recall (avoidance)
3	Saline	MeA	Saline	Recall (avoidance)
4	Saline	MeA	2-Dgal	Amnesia or recall
5	Saline	Water	Saline	Recall (pecking)

Figure 5.1; Schematic representation of the protocol of experiment 5.1.

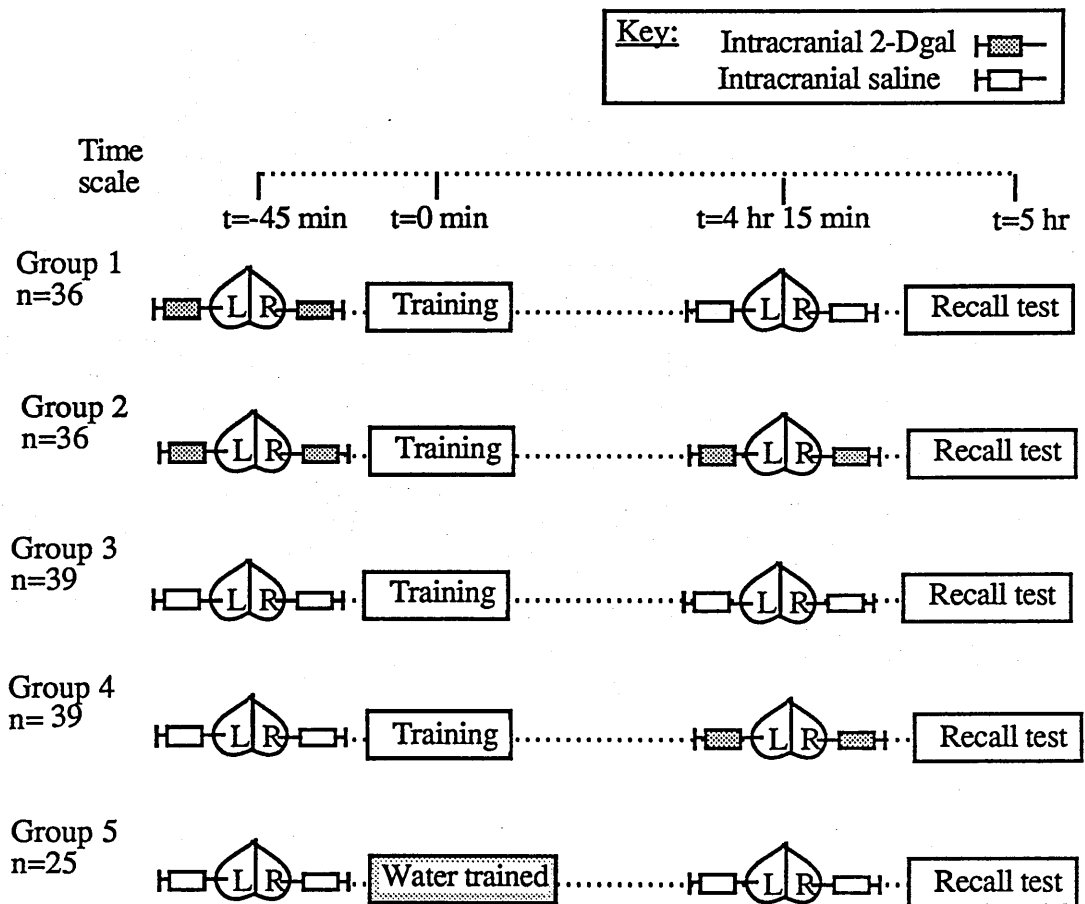


Figure 5.1; Five groups of chicks were injected i.c. bilaterally with either 2-Dgal or saline, 45 min before training and a similar time before testing. The fifth group acted as an extra control, being injected with saline on both occasions and trained with a water dipped LED rather than methylantranilate. Recall tests were carried out 5 hr after training and involved a serial 20 sec presentation of the red and green LED.

RESULTS

The results are illustrated in figure 5.2 and summarised in table 5.3. The data were expressed as mean peck latencies (MPL).

Table 5.3: Summary of the results from experiment 5.1, with Mean Peck Latency (MPL) for both the red and green LED and % avoiding scores for comparison.

Treatment	n	MPL Red	s.e.m.	% Av	MPL Green	s.e.m.	% Av
2-Dgal - Sal	36	12.41	1.38	50.0	13.39	1.29	52.8
2-Dgal - 2-Dgal	36	11.45	1.38	38.9	15.15	1.23	58.3
Sal - Sal	39	15.49	1.15	69.2	16.50	1.11	74.4
Sal - 2-Dgal	39	14.19	1.29	61.5	17.39	0.93	79.5
Sal - Sal (W-trained)	25	8.06	1.67	28.0	7.32	1.73	28.0

All possible comparisons were made between pairs of groups for the results of both the red and green LED. In the case of the red LED, group 2 (2-Dgal - 2-Dgal) had a significantly shorter peck latency than group 3 (Sal - Sal; $p=0.015$). Group 5 had a significantly shorter peck latency to the red LED compared with all other groups ($p\leq 0.018$).

The results of comparisons between groups for the green LED data are summarised in table 5.4.

Figure 5.2; Test of state dependent action of 2-Deoxy galactose in the colour-discrimination passive avoidance task.

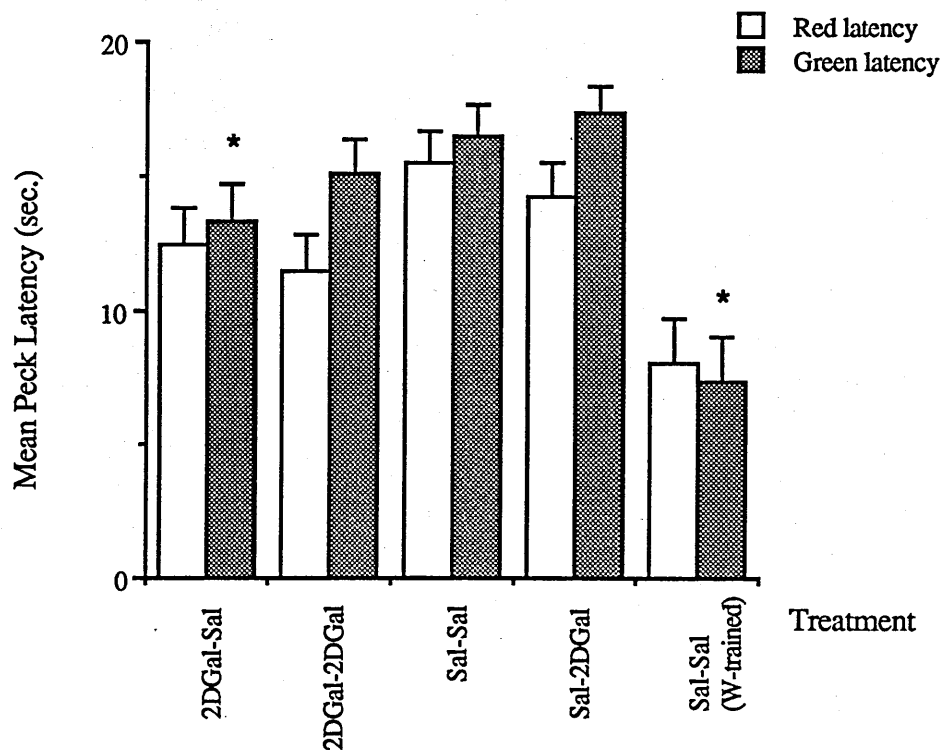


Figure 5.2; Five groups of chicks were injected bilaterally, i.e., with 2-Dgal or saline before training and testing. The mean peck latencies to both the red and green LED's are illustrated. The 2-Dgal - Saline group had a significantly shorter mean peck latency to the green LED compared with the Saline - Drug group ($p=0.015$) but not the Saline - Saline group ($p=0.084$). Also, there was a trend towards a shorter peck latency in the 2-Dgal - 2-Dgal group, compared to the Saline - 2-Dgal group ($p=0.069$).

Table 5.4: Summary of statistical comparisons between pairs of treatment groups for green LED data.

Comparison	p value
(2-Dgal - Sal) with (Sal - Sal)	p=0.084
(Sal - Sal) with (Sal - 2-Dgal)	-
(2-Dgal - Sal) with (Sal - 2-Dgal)	p=0.015
(2-Dgal - Sal) with (2-Dgal - 2-Dgal)	-
(2-Dgal - 2-Dgal) with (Sal - Sal)	-
(2-Dgal - 2-Dgal) with (Sal - 2-Dgal)	p=0.069

"-"= no significant difference.

The water trained group had a significantly shorter peck latency to the green LED than all other groups ($p \leq 0.009$). A comparison between group 1 (2-Dgal - Sal) and group 3 (Sal - Sal) yielded a p value of 0.084, and between group 1 (2-Dgal - Sal) and group 4 (Sal - 2-Dgal) gave a p value of 0.015, indicating that group 1 was amnesic, although this amnesia was not as pronounced as expected from previous results.

The comparison between group 3 (Sal - Sal) and group 4 (Sal - 2-Dgal) was not significantly different, indicating that there was no confounding effect on recall due to the second injection of 2-Dgal alone.

Comparisons between group 2 (2-Dgal - 2-Dgal) and group 1 (2-Dgal - Sal) yielded no

significant difference, as did a comparison between group 2 (2-Dgal - 2-Dgal) and group 3 (Sal - Sal). The comparison between group 2 (2Dgal - 2-Dgal) and group 4 (Sal - 2-Dgal) yielded a p value of 0.069. Thus, the peck latency of the crucial double drug group (group 2) lay somewhere between the peck latency of the amnesic group (group 1) and that of the recall group (group 3). There were no significant differences between sexes, in any of the treatment groups.

DISCUSSION

The results from comparisons are shown in table 5.4. For some reason, it was not clear that 2-Dgal induced amnesia after the first administration of the drug, since the Drug-Saline group did not have a significantly shorter peck latency to the green LED compared to the Saline-Saline group ($p=0.084$). This effect is probably due to an unexpectedly short peck latency in the Saline-Saline group. Normally the peck latency of the saline injected group would be between 18.0 and 20.0 sec, not the 16.5 sec recorded here. It is possible that the second injection of saline altered the chicks' performance in the recall task. There is some evidence to suggest that i.c. injection of saline can cause amnesia (Gibbs and Ng, 1977)

The peck latency of the Saline-Drug group was significantly longer than that of the Drug-Saline group ($p=0.015$) but not significantly different from the Saline-Saline group, indicating that the first injection of 2-Dgal did give rise to amnesia and that the second injection of 2-Dgal, just before recall, did not cause any profound abnormalities in the behaviour of the chicks during the recall test.

The crucial Drug-Drug group did not have a significantly different peck latency,

compared to either the Saline-Saline group or the Drug-Saline group. However, as pointed out earlier, the Saline-Saline group had a much shorter peck latency than normally expected. The fact that the peck latencies of the Drug-Drug and Drug-Saline groups were not significantly different, while those of the Drug-Drug and Saline-Drug groups were almost significantly different ($p=0.069$) is good evidence to suggest that 2-Dgal has not caused state dependent recall.

Experiment 5.2. Testing 2-Deoxy galactose for a state-dependent action in the sickness-conditioned aversion task.

INTRODUCTION

A review of 57 studies on state dependent learning (Eich, 1980) in which similar drugs were used, concluded that only 50% of the studies yielded positive results. One reason for the discrepancy between results from different studies, with similar drugs, may be that they utilised different learning tasks. It is conceivable that a drug may have a state dependent action in one learning paradigm but not in another. Since the present study has used more than one paradigm to investigate the behavioural effects of 2-Dgal, it was important to also perform a state dependence test using the sickness-conditioned aversion paradigm.

METHOD

49 day-old chicks of both sexes were separated into four experimental groups. All birds were trained by the method described previously; that is, they were allowed to peck at the green LED for 10 sec and injected intraperitoneally, 30 min later, with 0.1 ml of 1 mol

lithium chloride made up in 0.9% saline. All four groups were injected with LiCl since it was demonstrated previously, that this would lead to a subsequent avoidance of the green LED when compared to birds injected intraperitoneally with saline (Chapter 3).

The protocol for the experiment is displayed schematically in figure 5.3, along with the group n's. All groups were given 10 μ l bilateral intracranial injections of either 0.9% saline or 10 μ mol of 2-Dgal dissolved in saline, 10 min before being presented with the green LED and also 10 min before a similar presentation at the recall test. Those birds that did not peck at the green LED during the training period were rejected at the end of the experiment (30%). Group 1 (n=11) was given 2-Dgal before training and saline before testing, group 2 (n=14) was given 2-Dgal both before training and testing, group 3 (n=11) was given saline both before training and before testing and group 4 (n=13) was given saline before training but 2-Dgal before testing. The recall test took place four hours after training and involved single 10 sec presentations of the green LED and chrome bead, to each chick in turn. The i.c. injections, training and recall tests were performed "blind" by the author, while the i.p. injections were performed by D.B. Gilbert in the authors absence.

The results were expressed as % avoiding the LED and chrome bead. Analysis was carried out by individual Chi square comparisons between pairs of treatment groups.

Figure 5.3; Schematic representation of the protocol of experiment 5.2.

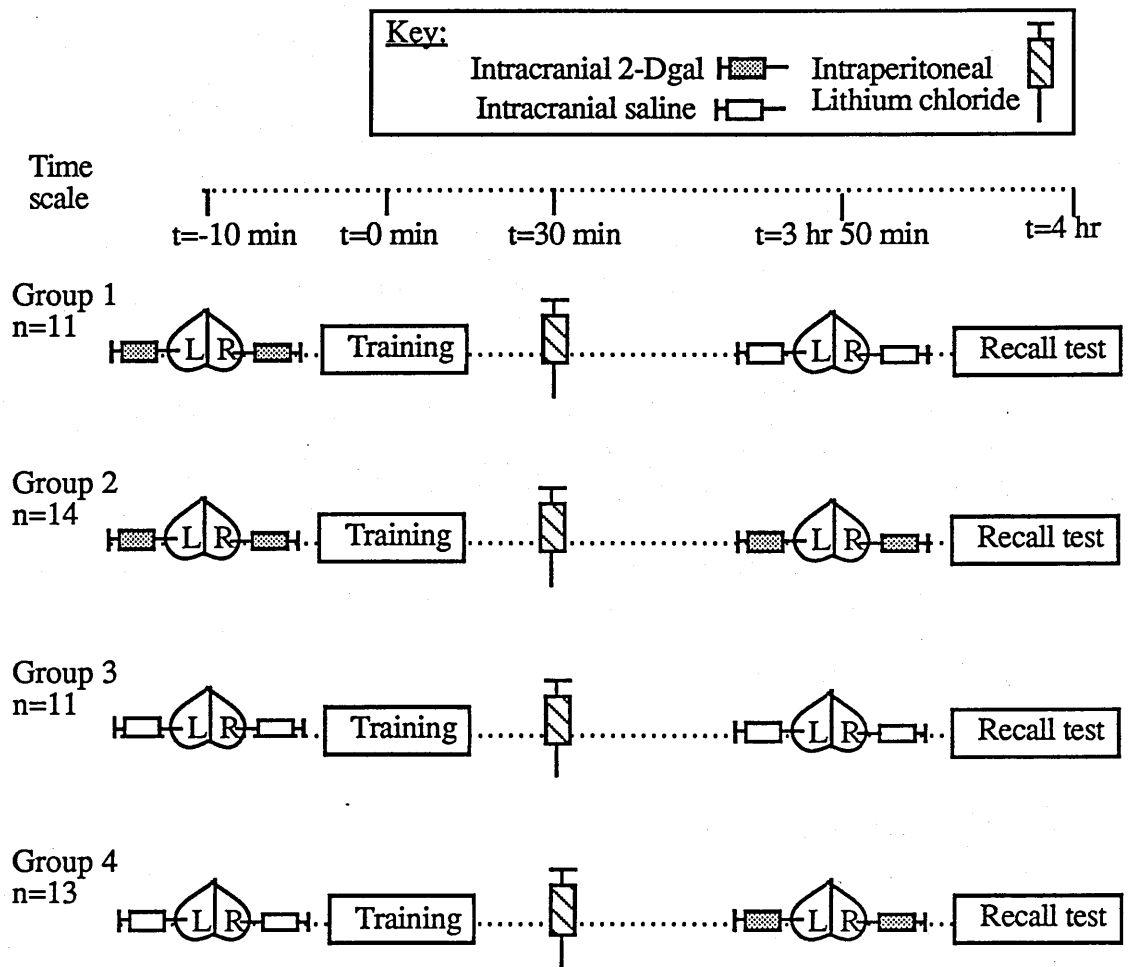


Figure 5.3; 49 day-old chicks of both sexes were separated into four treatment groups but given identical training. Chicks were injected bilaterally with either 2-Dgal or saline, 10 min before training and again before testing. Training involved a 10 sec presentation of the green LED followed 30 min later by an i.p. injection of LiCl. Recall tests were performed 4 hr after training. The results are illustrated in figure 5.4.

RESULTS

The results are illustrated in figure 5.4, in which the percentage of chicks avoiding the green LED for each of the four treatment groups is represented. Fewer chicks in the Drug-Saline group avoiding the green LED compared to the Saline-Saline group ($\chi^2=6.01$, $p\leq 0.05$) but not the Saline-Drug group ($\chi^2=1.607$). There was no significant difference between the Drug-Saline group and the Drug-Drug group ($\chi^2=0.00$). Also, there was no significant difference between the number of chicks avoiding the green LED in the Drug-Drug group and the Saline-Saline group ($\chi^2=0.97$) or the Drug-Drug group and Saline-Drug group ($\chi^2=0.671$).

Table 5.5: Summary of the results from experiment 5.2, expressed as percentage of chicks avoiding the green LED and chrome bead.

Treatment	n	% avoiding LED	% avoiding Chrome bead
2-Dgal - Saline	11	27.3	0.0
2-Dgal - 2-Dgal	11	36.4	27.3
Saline - Saline	14	64.3	28.6
Saline - 2-Dgal	13	61.5	27.1

The results illustrated in figure 5.4 and summarised in table 5.5 suggest that the amnesia was not caused by state dependence. Statistical comparisons between groups are summarised in table 5.6. Comparisons suggest that the first injection of 2-Dgal caused

amnesia, since 2-Dgal - sal and 2-Dgal - 2Dgal groups combined were different to the sal - sal and sal - 2-Dgal groups combined ($\chi^2=4.7055$, $p\leq 0.05$). A second injection of 2-Dgal, just before the recall test, did not lead to memory reactivation (2-Dgal - 2-Dgal vs 2-Dgal - sal: $\chi^2=0.21$). Although the 2-Dgal - 2-Dgal group was also be expected to be different from the sal - sal and sal - 2-Dgal groups, this was not the case ($\chi^2=0.97$; $\chi^2=0.07$, respectively). Nor was the comparison between the 2-Dgal - sal and sal - 2-Dgal groups ($\chi^2=1.61$).

Table 5.6: Statistical comparisons between individual treatment groups in experiment 5.2.

Comparison	χ^2	p value
(2-Dgal - Sal) with (Sal - Sal)	6.01	$p\leq 0.05$
(Sal - Sal) with (Sal - 2-Dgal)	0.06	NS
(2-Dgal - Sal) with (Sal - 2-Dgal)	1.61	NS
(2-Dgal - Sal) with (2-Dgal - 2-Dgal)	0.00	NS
(2-Dgal - 2-Dgal) with (Sal - Sal)	0.97	NS
(2-Dgal - 2-Dgal) with (Sal - 2-Dgal)	0.67	NS

In all groups less than 30% of the birds avoided the chrome bead, except the drug - saline group in which no birds avoided. There was no significant difference in the number of chicks avoiding the chrome bead across groups, however there was a trend towards fewer birds avoiding the chrome bead in the drug - saline group, compared with all other groups ($p\leq 0.089$).

Figure 5.4; Testing 2-Deoxy galactose for a state dependent action in the sickness-conditioned aversion paradigm.

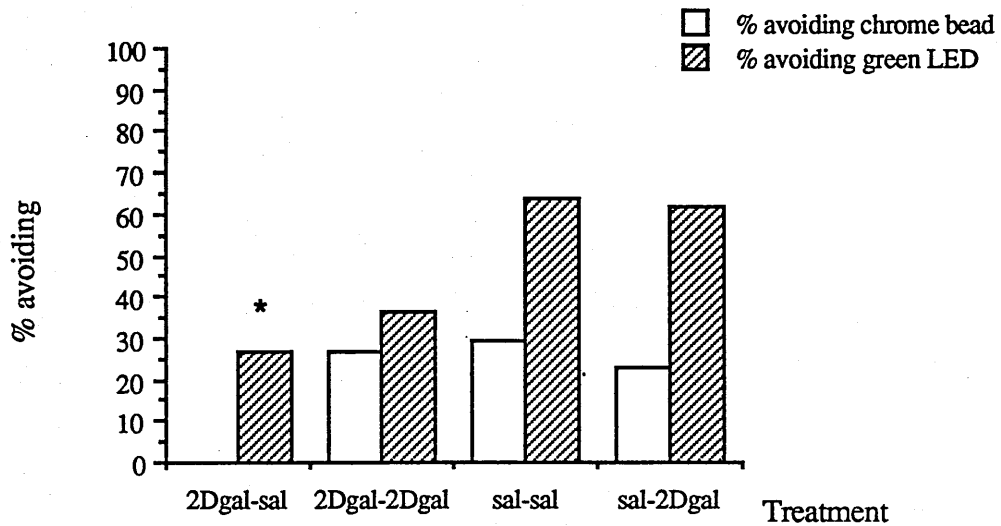


Figure 5.4; Four groups of chicks were injected with 2-Dgal or saline 10 min before training and a similar time before testing. The % chicks avoiding the green LED and chrome bead is shown. Fewer chicks in the 2-Dgal - saline group avoiding the LED, compared to the saline - saline group ($p \leq 0.05$). There was no significant difference between the 2-Dgal - saline and 2-Dgal - 2-Dgal groups.

DISCUSSION

Possibly the results from the sickness-conditioned aversion paradigm are vague for two reasons; firstly the total number of chicks used in each group was small because the experiment was performed as a control for a much larger study and secondly, the protocol required that each chick was handled and injected on three separate occasions, which may have caused a degree of stress and disorientation in the chicks. It is likely that this caused a greater variation in the behaviour of the chicks. There may also have been a degree of generalised avoidance of other stimuli, since the percentage of chicks avoiding the chrome bead was higher than expected from previous observation. Again, this may have been caused by the increased degree of stress associated with multiple injections.

In both experiments the performance of the control - drug groups was high, indicating that there was no symmetrical state dependence. The results from the control-drug groups of both experiments indicate that the drug had no effect on recall, apart from causing some chicks to become drowsy during the retention test. Administration of 2-Dgal before the retention test did not give rise to enhanced recall, although in both experiments, retention in the saline - saline group was lower than anticipated, perhaps because of the confounding effect of the double injection procedure. It should be noted that the results from both experiments are very similar, indicating that the effects measured were due to the administration of the drug and not to artifacts inherent in either of the learning paradigms.

The state dependence phenomenon has been cited as an example in the argument for the relative permanence of memory (Riccio and Richardson, 1984) in which a putative

learning experience is encoded as a long term memory almost immediately after training and remains completely intact but may be rendered inaccessible by drug or lesion (Meyer, 1984) rather than actually being prevented from forming. The fact that some amnestic drugs do appear to work in a state dependent fashion may lend weight to the argument that a memory can be "gone, but not forgotten," however, state dependence is the only reliable evidence that supports this school of thought. Furthermore, this theory does not take into account the action of those amnestic drugs that do not have a state dependent action.

It is important to seek amnestic drugs that have been proven to act in a specific fashion and not have a state dependent quality. Only then will the nature of the drug implicate the molecular processes needed for memory formation. It has been demonstrated previously that the protein synthesis inhibitor anisomycin, not only causes profound amnesia (Patterson et al, 1986) but also that it does so permanently, with no state dependence (Patterson, Rose and Bradley, 1989). The evidence reported here suggests that intracranial administration of 2-Deoxy galactose also gives rise to permanent amnesia that can not be reversed by a further administration of the drug. This implies that the addition the fucosylation of glycoproteins destined for incorporation into the plasma membrane is a metabolic step that is an integral part of the process of memory formation.

CHAPTER 6. GENERAL DISCUSSION AND FUTURE DIRECTIONS.

At the beginning of the thesis it was stated that one aim of the experimental psychologist was to collate and coordinate the observations of all other disciplines approaching the problem of memory and learning. This was, perhaps, an impossible goal for a PhD thesis. The aim of formulating a synthesis of all other findings at the many levels of analysis of memory seems as distant now as it was in the beginning. However, two new learning paradigms were designed and several important findings were made concerning the function of fucosylglycoproteins in memory formation. It is worth reviewing the work that has been achieved, while suggesting further work that could be carried out, given more time.

In chapter two, a serial colour-discrimination passive avoidance paradigm was created using a bicolour LED to present the chicks with two different coloured stimuli, one of which was paired with the bitter taste of methylanthranilate while the other was not. The important aspect of the paradigm was that the learning was expressed as a discrimination, or a change in the rate of expression of two responses, rather than a simple withdrawal of one behaviour. Thus it should be less likely that a change in behaviour, due to a side effect of some pharmacological treatment, was misinterpreted as learning. The initial goal of the paradigm was confounded by a number of factors. Firstly, young chicks were less willing to peck at the LED training stimulus, compared to the chrome bead, used previously. This may have been because the chrome bead acted as a super stimulus, by providing the chick with all the cues that it is prepared to peck at in order to find a source of water. This possibility was discussed in chapter two. A further problem of the colour discrimination paradigm was that, in spite of what has been reported on colour discrimination learning by Gibbs and Ng (1977), a significant proportion of the chicks

trained to avoid one LED stimulus would also avoid the other stimulus, exhibiting a strong generalised learning. This problem was overcome only by the fact that enough chicks in the population exhibited discrimination learning, giving a significant difference between the population means of the data from the paired and none paired stimuli.

The peck latency data was analysed by comparing the mean peck latencies for similar stimuli, between groups. Thus the mean peck latency to the green LED in one treatment group was compared with that of the control group. This method of comparison has the disadvantage of treating data collected from the presentations of paired and unpaired stimuli as independent, which is of course not the case. Furthermore, this method of analysis relies on making multiple pair-wise comparisons between groups. This has the obvious disadvantage of increasing the probability of making a type I error. At the time there seemed little alternative to this rather unwieldy method of analysis. If the peck latency measure were ignored and the data were expressed as % avoiding, then it may be possible to analyse data by factorial Chi square techniques as described in chapters three and four (Zar, 1984). It would be more ideal to use another quantitative measure of behaviour that provided a near-to-normal distribution of outcomes, thus enabling the possibility of multiple analysis of variance.

Gibbs and Ng have recently employed two different ways of analysing colour discrimination data (Crowe, Ng and Gibbs, 1989a, b). Firstly, the percentage of chicks exhibiting discrimination memory, expressed as:

$$\% \text{ discrimination memory} = 100 \times \frac{\text{Number avoiding paired and pecking unpaired stimulus}}{\text{Total number chicks}}$$

Secondly, the mean discrimination ratio, expressed by:

$$\text{Mean discrimination ratio} = \frac{\text{Number of pecks at unpaired stimulus}}{\text{Total number of pecks}}$$

Both methods of analysis give a single indicator of the proportion of chicks discriminating, by considering the presentation of paired and unpaired stimuli as dependent events. However, the data from chicks that pecked at the paired stimulus and avoided the unpaired stimulus was ignored (approximately 10% of the data). The data was ignored because it was unclear how to interpret this particular outcome in trained chicks. Crowe, Ng and Gibbs suggest that this outcome was a consequence of nonspecific effects of the treatments employed. However, no pharmacological treatment was employed in those papers, thus the outcome should be expected in around 10% of a normal population of trained chicks using this task. No matter what treatment is employed, there will always be a certain number of chicks in a trained population that will exhibit this outcome. It is highly undesirable to ignore any fraction of data collected from a normal population in the final analysis, simply because the data does not fit with expected behaviour.

Although the method recently used by Crowe, Gibbs and Ng is an improvement on previous approaches, what is needed is some method of analysis that includes all data and also treats the presentation of paired and unpaired stimuli as dependent events. The method of analysis must also provide some way of making a single comparison between two or more groups of chicks.

Since the data collected in this thesis is expressed as peck latency, ranging from 1 sec to

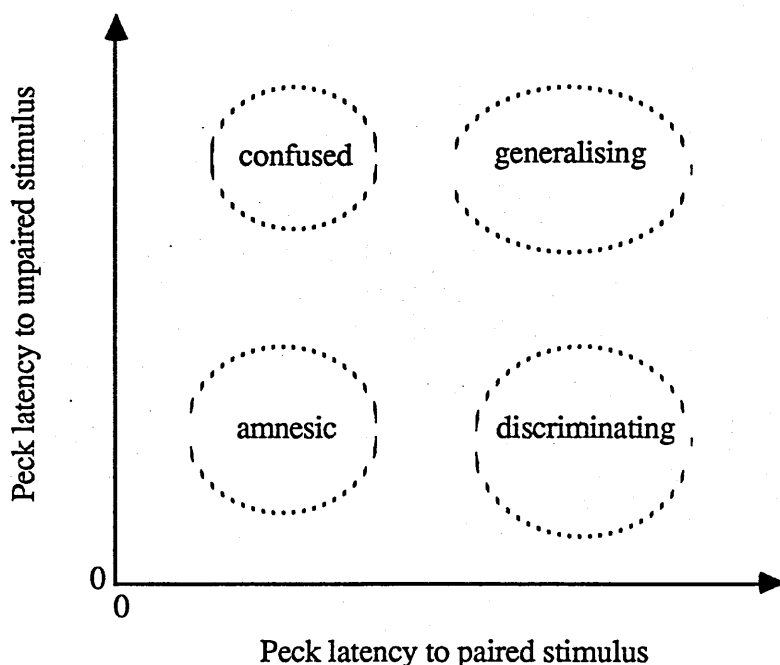
20 sec, there is the possibility of creating some ratio to take into account both the paired and unpaired data from each bird. The data could, for example, be processed by the method suggested by Crowe, Gibbs and Ng, to produce the "percentage discrimination memory" measure described earlier. This would generate a range of values from 0 (if the chick discriminated) to 1 (if the chick was confused). Chicks generalising or amnesic would generate a result that tended toward 0.5.

Alternatively the following ratio could be used:

$$\frac{\text{PL paired stimulus} - \text{PL unpaired stimulus}}{\text{PL paired stimulus} + \text{PL unpaired stimulus}}$$

The above formula would produce one value for each chick, in the range +1 to -1. A high positive value would indicate the individual had discriminated between the two stimuli while a very negative value would indicate the bird had exhibited confusion, by pecking the paired stimulus but avoiding the unpaired stimulus. However, both generalisation and amnesia would again be expressed by a score approaching 0. Somehow the ratio generated by observations from each chick would have to be plotted and populations compared. With both methods there is the possibility that real effects could be masked, by for instance, assigning the discrimination and amnesia outcomes with similar ratios. It is also difficult to suggest what type of analysis could be applied to data expressed in this way.

One last suggestion for an improved way of treating data from colour discrimination paradigms is to express the distribution of individual outcomes as follows:



The distribution of the four possible behavioural outcomes can be represented as above, by plotting the peck latencies to the paired and unpaired stimuli on horizontal and vertical axes. The distribution of outcomes in two populations of chicks, say drug and saline treated groups, could be compared by cluster analysis. There is, however, no guarantee that any more complex system of analysis will be better than the one applied to the data in the thesis. It should also be noted that labels such as "discriminating" and "generalising" inflict experimental interpretation on data even before it has been collected.

The findings of the experiments described in chapter two indicated that 2-Deoxy galactose caused amnesia for the colour-discrimination task, when injected in one administration, 45 min before training. Pair-wise comparison of the data indicated that the amnesia developed slowly, between 1 and 2 hr after training, indicating that the process inhibited

by 2-Dgal was required for the formation of long term, rather than short term memory. However, overall comparison of data indicated that there was no variation in observations over time.

The time window in which 2-Dgal is effective is longer than those of the protein synthesis inhibitors. The drugs, cycloheximide and anisomycin, were reported to cause amnesia when injected between 30 min before and 30 min after training (Gibbs and Ng, 1977). Emetine, however, was only effective when injected between 15 min before and after training (Patterson et al, 1986). The evidence suggests that 2-Dgal causes amnesia in a similar way to the protein synthesis inhibitors but has a longer time window in which it is effective. It may be that the estimation of the effective time window of 2-Dgal is inaccurate (Rose and Jork, 1987) since the study used a double administration technique. 2-Dgal was injected both before and after training and the time of administration was estimated as the mid point between injections. The results reported in chapter two suggest that the double injection technique was unnecessary, thus a further experiment could be carried out to make a more accurate estimate of the effective time window of 2-Dgal, using only one administration of the drug.

The results of the dose response experiment in chapter 2 were inconclusive, since it was not clear why the higher doses of 2-Dgal did not appear to cause amnesia. This experiment could be repeated with a two-stimulus discrimination paradigm and a greater range of doses of the drug, in order to determine whether 2-Dgal really does have a U-shaped dose-response effect, or whether the effect was due to a behavioural side effect of large doses of the drug.

In chapter three a sickness-conditioned aversion paradigm was created and the effect of 2-

Dgal was tested. In the paradigm, day-old chicks were caused to acquire an aversion to a stimulus that they had previously pecked, by sickness caused by lithium chloride injection. The important aspects of this paradigm are: i) that a sickness-conditioned aversion was acquired to a stimulus that did not include any gustatory or visceral cues, implying that the LED or chrome bead was interpreted as food by the chick; ii) the chicks were prepared to make an association between a CS and US that were separated in time by 30 min, implying that some short term-memory for the visual attributes of the CS was maintained without reinforcement during this period.

2-Dgal induced amnesia for the sickness-conditioned aversion task, indicating that glycoprotein fucosylation was required for memory formation. The fact that injection of 2-Dgal after the CS but before the US did not cause amnesia is open to interpretation, since the time window during which 2-Dgal is effective lasts for 2 hr or more. One interpretation is that fucosylglycoproteins are required for the memory of the CS during the 30 min period between CS and US. This could be tested further by a study in which the i.c. injection of 2-Dgal was administered at various times from 2 hr before training, to 2 hr after the injection of lithium chloride.

Much more work on the behavioural parameters of the sickness-conditioning task could be carried out in the future, since there are many unanswered questions concerning this task. For instance, how long does the conditioned aversion last after training and how quickly does it develop? To answer this question a number of groups of chicks could be given retention tests at various times after the lithium chloride injection, from 2 hr up to 24 hr. Also, are chicks prepared to learn the association between sickness and other sensory modalities? For instance, sickness could be paired with flavour, or even sound, as well as colour. If this type of sickness-conditioning were possible, an even stronger

learned response may be initiated in the chicks. If this were the case, the intensity of sickness required for learning could be reduced, by reducing the dose of lithium chloride. Also, it may be possible to extend the time delay between CS and US, which would be of great use in the further study of pharmacological effects on sickness-conditioned aversion.

What other drugs will cause amnesia for the sickness-conditioned aversion task? It is likely that protein synthesis inhibitors will cause amnesia for the task, since this has been achieved with rodents (Tucker and Gibbs, 1979) but it would be possible to learn more about the dynamics of the short-term memory between CS and US by using other inhibitors such as glutamate, ouabain and AP5. Does sickness-conditioned aversion have similar biochemical and morphological correspondents as passive avoidance learning? The task could be used in conjunction with the techniques that have been used previously, such as electronmicroscopy and electrophysiology, to determine the cell biology correspondents of training. Finally, does this type of learning require similar brain nuclei as the passive avoidance task? A study using the metabolic marker, 2-Deoxy glucose, following training, would yield an interesting answer.

The results of chapter four suggest that 2-Dgal, injected before training, will cause amnesia for the passive avoidance task when injected into the right forebrain hemisphere but not the left. The result fits well with previous biochemical findings, that fucose incorporation is highest in the right forebrain base after training (Mc Cabe and Rose, 1985). There is however, little to explain why glycoprotein fucosylation should be necessary in the right hemisphere, more than the left. Clearly, the left hemisphere is not redundant in the passive avoidance task because so many other studies have implicated the left hemisphere as more important than the right. So the result should not be assumed to indicate that memory formation requires a certain molecular process in one hemisphere

but a different molecular process in the other. This would imply that there is more than one molecular mechanism of memory formation. I feel that it would be a dangerous assumption to interpret the result in this way. It is more reasonable to suggest that the 2 hr pulse of 2-Dgal, injected 45 min before training, has inhibited a process of memory formation at a time after training in which the right hemisphere plays a greater part. It could be that the fucosylation of glycoproteins in the left hemisphere plays a greater role in memory retention at other times after training. This could be tested by varying the unilateral injection of 2-Dgal, before and after training.

The increase in fucose incorporation in vitro (McCabe and Rose, 1985) was greatest in the right forebrain base 3 hr after training and was not evident 24 hr after training. This result seems to imply that fucose incorporation is required for some part of memory formation but not its maintenance. The behavioural findings reported in chapter four suggest that once the fucose incorporation is inhibited in the right hemisphere, amnesia is permanent, implying that fucosylation is a necessary process that enables memory formation to take place but is not evident 24 hr after training. Thus, it could be that fucosylglycoproteins are a necessary part of memory formation, but are not required for the continued maintenance of that memory representation.

The results of the second experiment of chapter four indicated that sickness-conditioning could not be inhibited by injection of 2-Dgal into any one forebrain hemisphere alone. This suggests either, the locus of memory for this task is more diffuse throughout both forebrain hemispheres, or that there is more than one area of the forebrain, capable of learning the task. The result also indicates that there are at least two mechanisms for memory storage of information concerning bead pecking, in the chick. A further experiment could be carried out by combining unilateral injection of 2-Dgal (or other

drugs) with an eye patch technique, in which one eye was occluded during training. The outcome predicted for this experiment would be amnesia in both the left eye occluded and right eye occluded conditions.

The results reported in chapter five, in which 2-Dgal was tested for a state dependent action in both learning paradigms, indicated that the drug did not cause amnesia in this way. Thus it was concluded that the amnesia caused by 2-Dgal was directly due to its molecular action on glycoprotein fucosylation, rather than to the dissociation of learning by a non-specific effect of the drug such as sickness or state alteration. There is little more that can be said about these results except that the phenomenon of state dependence is rarely considered in the study of behavioural pharmacology of memory. In future, drugs used in the study of memory should be tested for state dependent qualities as a matter of course.

The fact that two new paradigms of learning in the young chick have been developed and reported here does not leave room for complacency. As mentioned in chapter one, the only way to fully understand the mechanism (or mechanisms) of memory is to study many learning paradigms and to compare the similarities of neuronal changes after learning. There is room, I believe, for developing a spatial task using chicks, which may require heat reinforcement, social reward or escape from water (chicks can swim for short distances, in warm water!). There is also the possibility of an operant learning task in which a social reward or heat reinforcement is used. This type of task has been used previously with the pressing of a floor pedal as the operant and a view of an imprinting stimulus as reinforcer (Bateson and Reese, 1969). Although it may be unwise to use an imprinting stimulus as a source of reinforcement to study operant conditioning, since the effects of two different types of learning may be confused, it would be possible to create such a task using a heat reinforcement.

Not only must the problem of memory be approached by studying many learning paradigms, but also as stated earlier, must be approached on many different levels by different disciplines. The value of the levels approach will emerge with time, once workers on each level of analysis learn to translate their findings between levels.

In final summary, it can only be reiterated that the drug, 2-Deoxy galactose, causes amnesia which develops slowly after training and therefore interferes with a consolidation process, not acquisition of learning. It is likely that 2-Dgal interferes with memory consolidation by its direct molecular action on glycoprotein fucosylation. This implies that glycoprotein fucosylation has a key role in memory consolidation. Since the fucosylglycoproteins are exclusively destined for incorporation into the synaptic plasma membrane it makes sense to suggest that the mechanism of memory storage involves an interaction between fucosylglycoproteins and the synaptic plasma membrane (along with other unspecified factors). The type of interaction taking place may be one of the following:

- i) the fucosylglycoprotein makes permanent the plastic changes in the synapse, by strengthening or making permanent, the changes in synapse morphology;
- ii) the fucosylglycoprotein makes possible plastic changes in an otherwise non-plastic synapse, by changing the plastic properties of the synaptic plasma membrane;
- iii) the fucosylglycoprotein increases the "weighting" of a synapse by bringing about a stronger signal strength between cells, by mechanically drawing together two membranes like a "hook and line" system.

Although it would be difficult to test these hypotheses using a purely behavioural approach, my own tendency is towards the first of the above suggestions. The clinical

evidence suggests that an increase in fucosylglycoproteins, or rather, the inability to metabolise the molecules, is characterised by severe mental retardation and neuronal degeneration, in the disease, fucosidosis (Patel, Watanabe and Zeman, 1972). It is possible that those symptoms could be the direct result of very low levels of synaptic plasticity, due to an over deposition of fucosylated glycoproteins at the synaptic plasma membrane.

Obviously there are more experiments to be done using 2-Deoxy galactose. However, I believe that only a certain amount can be learned by the purely behavioural techniques that have been employed here. In the future the most valuable experiments to be carried out will be to determine the effect of 2-Dgal injection on cell biology after training. Does the drug, for instance, prevent the changes in spine morphology after passive avoidance training? Similarly, will 2-Dgal prevent "bursting" and the morphological changes at the electron-microscopic level? If it is the case that fucosylglycoproteins somehow facilitate the plastic changes of memory formation in the synapse it is likely that the answer to these questions will be in the affirmative.

APPENDICES

1. Housing and training conditions.

Ross I Chunky chicks were hatched in our laboratory and held in communal incubators until 24-36 hr post-hatch (38-40 °C, 12 hr light /dark cycle). On the morning of each experiment, chicks were taken in a group from the incubator to the training room and placed in pairs in aluminum training pens. Each pen was 20 by 25 by 20 cm and was placed on top of a clean sheet of blue paper towel. Chick starter crumbs were scattered on the floor of each pen before the chicks were placed there (except for the sickness-conditioning experiments). Each pen was illuminated by a red 25 W light bulb while the main laboratory lights were kept off, making it more difficult for the chicks to be distracted by activity in the laboratory. One chick in each pair was identified by a spot of animal dye, placed on its back before the chick was introduced to the training pen. After being placed in the training pens the chicks were left undisturbed for 1.5 hr in order to allow them to acclimatise.

2. Construction of the LED training lures.

Two identical training lures were constructed. The first was always used for pretraining and retention trials, while the second was used for training trials only. Thus, only one LED lure was contaminated by methylantranilate.

The bicolour LED was fixed at the end of a hollow perspex rod which contained wires connecting the LED with a 9 V battery, resistor and three way toggle switch, housed in an oblong die-cast metal casing (8.5 by 3 by 3 cm). All parts were purchased from RS

Components LTD. The battery, resistor and toggle switch were wired in such a way that the middle position of the switch was used as the "off" position while the other two settings illuminated the LED red and green.

3. Unilateral injection of [^3H]-2-Deoxy galactose.

In order to demonstrate that 2-Deoxy galactose, when injected intracranially into one hemisphere only, did not significantly diffuse to the contralateral forebrain hemisphere, [^3H]-2-Dgal (5 μCi) was injected intracranially in a dose of 10 μmol (as in the experiments reported in Chapter 4) into either the left or right forebrain of 20 chicks. The chicks were killed either 1.5 hr ($n=8$) or 3 hr ($n=12$) after injection. Also, 2 chicks were injected with saline and killed 3 hr later. The forebrains were removed, carefully separated into left and right hemispheres and placed in test tubes cooled in ice. Each hemisphere was then homogenised with 2.5 ml distilled water. Three 0.5 ml samples of tissue were taken for each homogenate. Each sample was added to a scintillation vial along with 1 ml Protosol, 8 ml of scintillant and 0.5 ml distilled water. The radioactivity of each sample was then counted.

The data is represented in figure 6.1. The data was analysed by comparing the counts per minute in the injected and non-injected hemisphere, using the Student's T test. The amount of radiation in the injected hemisphere was significantly greater than in the non-injected hemisphere, 1.5 hr after injection ($t=3.94$, $p\leq 0.001$) and 3 hr after injection ($t=4.18$, $p\leq 0.000$).

The technique used was similar to that used by Howard, Rogers and Boura (1980) to measure the amount of contralateral diffusion of unilaterally injected glutamate. Rogers

Figure 6.1; Unilateral injection of [³H]-2-Deoxy galactose.

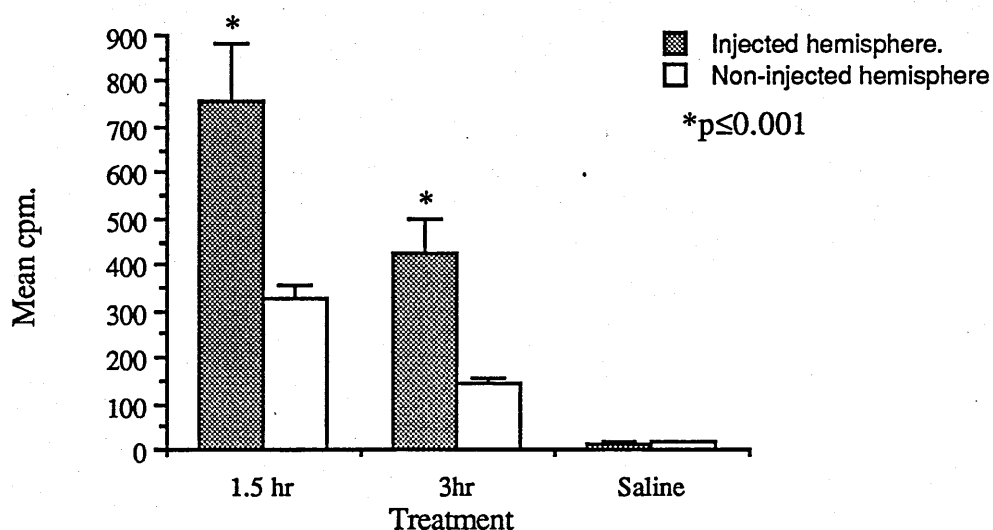


Figure 6.1; Tritiated 2-Dgal was injected i.c. into either the left or the right hemisphere of 20, day-old chicks. The chicks were killed 1.5 hr later (n=8) or 3 hr later (n=12). Also, two chicks were injected with saline and killed 3 hr later. Three samples from each hemisphere were counted and the average counts per minute from each hemisphere were compared by a Student's t test. The amount of [³H]-2-Dgal in the injected hemisphere was significantly greater than in the non-injected hemisphere, both 1.5 hr and 3 hr after injection ($p \leq 0.001$).

also found that the amount of radioactivity found in the non-injected hemisphere was significantly different from that found in the injected hemisphere. The results should, however, be treated with some caution for two reasons. Firstly, the amount of radioactive drug found in the non-injected hemisphere was higher than control levels, so a small amount of drug passes into the contralateral hemisphere. It is not known what dose this represents. Secondly, this result should not be assumed for other drugs since different drugs may have different diffusion properties.

4. Testing the effect of 2-Deoxy galactose on locomotor activity.

During earlier experiments with the usual bilaterally injected dose 2-Deoxy galactose (2-Dgal), 20 μmol , (as used by Rose and Jork, 1987), one or two chicks were noted to be more active than the others, a few minutes after injection. Their behaviour was characterised by repeated "peeping" and increased locomotor activity. In order to determine whether this increased locomotor activity was due to the injection of 2-Dgal, a simple locomotor activity test was designed and performed for five doses of 2-Dgal along with three control treatments.

A 2 \times 2 grid was placed on white paper, on the floor of each training pen, so that the pen floor was divided into 4 similar sized rectangles (each 10 by 12.5 cm). Chicks were placed in pens in pairs, as usual, with chick crumbs scattered on the floor of each pen. The chicks were then left to acclimatise for the usual period of 1.5 hr.

At the beginning of the experiment, each chick was injected with a total dose of 10 μmol (n=12), 20 μmol (n=12), 40 μmol (n=12), 100 μmol (n=10) or 200 μmol (n=10) of 2-Dgal by the usual method, or was given one of three control treatments: bilateral injection

of 0.9% saline (n=14); handling but no injection (n=14); or no handling (n=12). After treatment the chick was left alone for five minutes and then observed for five minutes. During the observation period the number of times the chicks crossed completely from one quarter to another, was counted. Diagonal crosses were recorded as one crossing. Although this counting method was biased, since chicks that spent more time in the center of the pen would be given a higher score than those spending longer at the perimeter, the period of observation was considered to be long enough to reduce this bias to acceptable levels.

Treatment administration was staggered at 5 min intervals, so the time between treatment and the onset of the observation period was always 5 min. The marked chick in each pair was always treated and observed first. The protocol was repeated over four days, giving a total n of 96. The sex of each chick was recorded at the end of the experiment.

A factor analysis of variance was performed on the data, followed by a Duncan's multiple range test, using the SPSSx package on the Walton Hall VAXcluster mainframe computer. This method of analysis enables all comparisons between pairs of treatment groups. The data were expressed as mean activity scores and are represented in figure 6.2. The p values given in the figure refer to comparisons between individual treatment groups and the saline-injected control group. There was no significant difference between the sexes ($p=0.86$), between the marked and unmarked chicks ($p=0.87$) or between any of the three control groups (which had average activity scores of 6.9, 6.2 and 6.1 crosses in 5 min, respectively). Injection of a dose of 40 μmol or more of 2-Dgal produced significantly more locomotor activity compared to the injection of saline ($p\leq 0.05$). Although the injection of a 20 μmol dose of 2-Dgal (similar to that used in the experiments reported earlier) produced a mean activity score of 12.75, this was not

significantly greater than the mean activity score of the saline-injected group (6.9).

The results indicate that intracranial injections of 2-Dgal cause an increased locomotor activity. This may indicate that the drug causes increased arousal. The result conflicts with observations by Rose and Jork (1987), that noted a drowsiness during training, in those chicks injected with 10 μ mol 2-Dgal before training. Since chicks were observed 5 min after injection in the present experiment, while Rose and Jork trained chicks 10 min after injection of the drug, it may be possible that the initial increase in activity is immediately followed by a short period of decreased activity. This could be tested using a similar procedure to the one described above, by changing the time of onset of the observation period with respect to the time of injection.

Figure 6.2; Locomotor activity of chicks after various treatments, including injection of 2-Deoxy galactose.

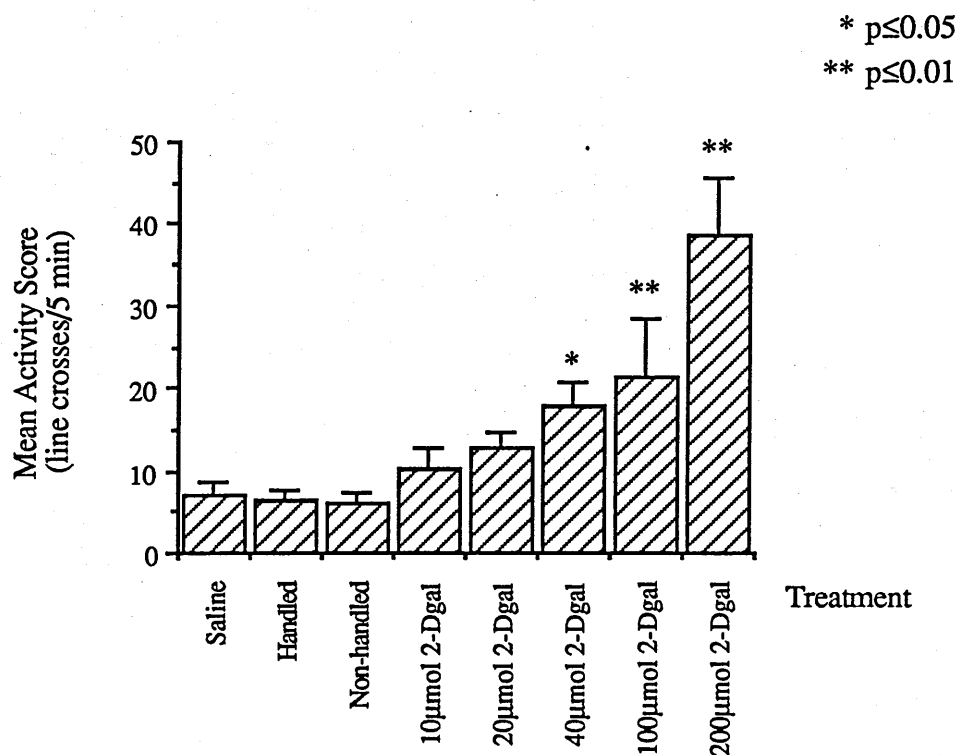


Figure 6.2; Mean locomotor activity, measured by the number of line crosses on a 2×2 floor grid, was recorded after eight different experimental treatments. Each treatment was administered 5 min before the beginning of the observation period, which lasted for 5 min. The error bars represent standard error mean. All possible comparisons between individual pairs of groups were made, using the Duncan's multiple range test. The groups injected with 40, 100 and 200 µmol 2-Dgal were significantly more active than the saline injected controls.

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